INVOLVEMENT OF LUNG DENDRITIC CELLS

DURING EARLY STAGES OF BREAST CANCER LUNG METASTASIS

by

Luda Lin

A thesis submitted to Johns Hopkins University in conformity with the requirements for

the degree of Master of Science in Engineering

Baltimore, Maryland

May 2020

Abstract

Breast cancer is one of the most pervasive malignancies in women in the world. Metastasis, the spread of malignant cells from a primary tumor to distant organs, is the main cause of death by cancer. The immune system interacts with cancer through its whole development processes, with its members, according to their phenotypes, involved in either suppression or support of malignant cells. Dendritic cells are significant in this response because of their roles as professional antigen-presenting cells. Their role is to initiate immune responses against cancer through the activation of the adaptive immune system. However, much less research has been done on the role of dendritic cells in metastasis, especially in early metastatic colonization. Dendritic cells secrete extracellular vesicles, particles carrying multiple molecules like proteins, microRNAs, lipids and metabolites inside or on their phospholipid bilayers, during antigen presentation and in development of cancer. However, currently we do not know the role of extracellular vesicles secreted by dendritic cells in response to metastasis. Here, we tried to investigate the involvement of lung dendritic cells during early stages of metastatic seeding of lungs. We injected tdTomato+ mammary tumor cells into wildtype mice and dissect lungs from them after certain time points. We then analyzed sections of lungs stained with an antibody against CD11c, the classical marker of dendritic cells. We found CD11c+ particles in metastases. We then testified whether those particles were really released by dendritic cells by staining them with CD24, a marker used to distinguish between dendritic cells and CD11c+ macrophages, showing that those particles were not released by dendritic cells. Besides characterizing particles, we also attempted to quantify dendritic cells surrounding metastases at different time points and comparing labeling of dendritic cells with CD11c only or with both CD11c and CD24. We cannot draw a definite conclusion about whether lung dendritic cells are involved in immune responses against infiltrating metastatic cells and more research is required for further investigation.

Thesis Readers

Dr. Andrew J. Ewald

Dr. Takanari Inoue

Dr. Jian Liu

Acknowledgements

Hereby I would like to show my sincere gratitude to everyone who has helped me get through the master program and the work relevant to the thesis. Firstly, I am extremely grateful to everyone in Ewald Lab. I first got to know the work done by Ewald Lab when I was doing literature review for my undergraduate project, when I was quite fascinated by the paper about polyclonal metastasis by Dr. Kevin Cheung. What a providence it was that I finally joined the lab. I would like to thank Dr. Isaac Chan, who provided important guidance for me not only in technologies relevant to experiments and graduate program application, but also how to behave as a graduate student and to think like a graduate student in the future. It does not mean that our relationship has always been smooth, but I am very thankful for his guidance and lessons taken from the lab experience. I also want to thank Gayathri Ramakrishnan and Juan Carlos Raminez, who guided me to be members of the lab, in academics and in life. I hope that they have a good life in Wisconsin and Texas respectively. I also want to thank Dr. Eloise Grasset, who provided some important advice in experiments. I am also grateful to Matthew Dunworth, whose technologies of high-content analysis really surprised me at the very beginning and becomes the closest member of the lab for me later in the lab. Finally, I want to thank Andy, who is perceptive in both research and lab management. He was already prophetic when I was interviewed by him. Although we do not have too many chances of direct contact, when we had talks, he always gave important advance which proved correct to me. He sets a model of a successful and helpful principal investigator managing a successful lab in such a highly competitive world of academia for me. I also want to thank other personnel working for JHMI, such as Linda, who has always been very friendly and helpful.

I also want to thank friends I made in the BME MSE program, such as Yuchen Ge and Ruitao Zhang. It can be very difficult for various reasons sometimes for a student learning abroad. We talk about difficulties we have confronted and help each other get through. I am very grateful for such a mutually supportive friendships we have formed.

I also want to show my gratefulness to the BME department because of the two-year thesis-based MSE program they provide. It functions like a “trial version” of a PhD program for me, letting me know how the life of an academic career is and allowing me to choose whether I follow this path without any cost.

Lastly, I would like to thank my parents, who have been unconditionally supportive of me before and after I entered Johns Hopkins, materially and emotionally.

Table of Contents

List of Tables

List of Figures

Table of Abbreviations

ACUC	Animal Care and Use Committee
AF647	Alexa Fluor 647
ARF6	ADP-ribosylation factor 6
BSA	bovine serum albumin
CCL2	chemokine (C-C motif) ligand 2
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
cDC	conventional dendritic cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNase	deoxyribonuclease
DPBS	Dulbecco's phosphate buffered saline
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
EV	extracellular vesicle
FBS	fetal bovine serum
GFP	green fluorescence protein
HER2	human epidermal growth factor receptor 2
Hsp70	70 kilodalton heat shock proteins
IFN-γ	interferon gamma
IL	interleukin
K-14	cytokeratin 14
MDSC	myeloid-derived suppressive cell
MHC	major histocompatibility complex
MIC A/B	MHC class I polypeptide-related sequence A/B
MMTV	mouse mammary tumor virus
MV	microvesicle
MVB	multivesicular body
NK cell	natural killer cell
OCT	Tissue-Tek OCT Compound
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PGE2	prostaglandin E_2
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
PyMT	polyomavirus middle-T antigen

ROS	reative oxygen species
TAM	tumor-associated macrophage
TCR	T-cell receptor
TGF-β1	transforming growth factor beta 1
TME	tumor microenvironment
TNFR	tumor necrosis factor receptor
Treg	regulatory T cell

Introduction

Breast Cancer

Breast cancer is one of the most common malignancies in women. At least one in ten women may suffer from breast cancer [1]. Although its mortality rate has decreased by about 40% in the United States in the past 30 years, breast cancer is still the second most common cause of death by cancer for women in this country [2].

Breast cancer is commonly classified into five intrinsic molecular subtypes: luminal A, luminal B, HER2-enriched, basal, and normal-like [3]. They are identified according to three classical immunohistochemistry markers, ER, PR and HER2, and proliferation is measured by Ki67 [4]. Luminal A and normal-like are marked by ER+PR+HER2-Ki67-; luminal B can be divided into two types: ER+PR+HER2-Ki67+ and ER+PR+HER2+Ki67+; HER2-enriched is identified as ER-PR-HER2+Ki67+; Basal is labeled as ER-PR-HER2-, and thus called triple-negative [5]. There is great diversity in prevalence and prognosis between five molecular subtypes: Luminal B and A are most common; prognosis of HER2-enriched, basal and ER+PR+ HER2+Ki67+ Luminal B subtypes is worse than other subtypes [6]. Different gene expression profiles of the five subtypes determine different therapies for optimal treatment [4].

Metastasis

Metastasis is the spread of malignant cells from a primary tumor to one or more distant sites. It is the last stage of cancer and the main cause of death. While early stage breast cancer can be relevantly easier cured by local therapies such as surgery, metastatic breast cancer is still considered incurable. However, many therapies, including chemotherapies and immunotherapies, are being developed to treat metastasis [4], [7]. While the 5-year survival rate of early stage breast cancer was as high as about 90%, this figure fell to less than 30% for metastatic stages, according to data between 2009 and 2015 [8].

Metastasis can be divided into five stages: invasion, intravasation, circulation, extravasation, and secondary-site colonization. Invasion is dissociation of individual cells or groups of cells from a primary tumor to surrounding tissue [9]; intravasation is dissociated cells entrance into blood or lymph vessels through the endothelium of the two types of vessels [10]; circulation is that of malignant cells through the circulatory system [11]; extravasation is circulating cells' exit from blood vessels via the endothelium to enter secondary sites [12]; secondary-site colonization is the formation of metastatic tumors by seeded cells in distant sites [13][14]. It is widely believed that metastasis occurs through those five steps in a timely manner, although there are some disagreements stating that metastasis is independent of local invasion [15]. Although those stages involve different cell activities, some common properties shown by metastatic cells during the whole processes of metastasis have been summarized, from which heated debates also arise in academia. Some examples of those common properties are shown below.

Metastasis has been believed to be carried out by individual cells, such as individual dissemination from primary tumors and colonization and growth of metastases by and from individual circulating malignant cells [16]. However, this observation has been challenged by multiple clinical and experimental observations that clusters formed by more than one malignant cell disseminate from primary tumors [17], circulate in blood vessels [18], and colonize secondary organs [19]. Cheung et al. showed that in PyMT-MMTV mice, a mouse cell line commonly used for breast cancer research, more than 95% metastases in lungs were developed from clusters rather than individual cells. He also showed that clusters were much more advantageous in colony formation in vitro, compared to individual cells [19].

Most cancers, including breast cancer [20], show intratumoral heterogeneity (reviewed in [21]), which means that they are composed of multiple subpopulations with different phenotypes. Different subpopulations are diverse in their abilities, including those to invade and to resist therapies [21]. Certain subpopulations have been found to be more invasive and take dominant roles in metastasis. In

tumors in multiple breast cancer mouse models, a subpopulation expressing keratin-14 (K14), an intermediate filament protein and a marker for the basal state, although comprising a small proportion in primary tumors, were found to lead cancer invasion and dominate circulating tumor clusters and micrometastases just after colonization. However, its proportion declined to the primary tumor level as metastases grew [17], [19].

Tumor cells have strong plasticity and are believed to experience phenotypic changes before and during metastasis so that they can be successfully seed in secondary organs [22]. Epithelial-mesenchymal transition (EMT) is a widely believed model about such phenotypical changes considered necessary for metastasis: in carcinomas, or cancers starting from epithelial tissues, malignant cells have to suppress expression of genes denoting epithelial phenotypes and promote expression of those denoting mesenchymal traits to increase their invisibility by decreasing cell-cell junctions and strengthening mechanical interactions between cells and extracellular matrices (ECM), and after successfully colonizing secondary organs, metastatic cells have to reverse to epithelial phenotypes again, i.e., mesenchymal-epithelial transition (MET) (reviewed multiple times, such as in [23], [24]). However, the necessity of EMT in metastasis is challenged in multiple cancer types such as breast cancer [25], [26]. Some research shows that EMT is dispensable for metastasis but promotes resistance against chemotherapy of breast cancer lung metastasis [26]. Some research shows that EMT is even detrimental to metastasis because it leads to vulnerability to apoptosis induced by reactive oxygen species (ROS) [27]. The concept of EMT also keeps evolving, changing from a switch-like transition between epithelial and mesenchymal states to plasticity in a continuum between the two states, including multiple intermediate states (or “partial-EMT” states), in which epithelial markers may not be downregulated when mesenchymal ones are upregulated [28]. In conclusion, the detailed mechanism of involvement of cancer cell plasticity in metastasis is still disputed.

Our research focuses on colonization, which, as mentioned above, is the last stage of metastasis. In this stage, metastatic tumor cells leaving the circulation system through extravasation are seeded in distant sites from primary tumors. After leaving blood vessels, malignant cells still need to go through multiple steps such as infiltration into secondary organs, evasion from the local immune system and other host defense mechanisms, settlement in supportive niches for survival, entry into dormancy, and outburst [13]. Compared with the microenvironment of a primary tumor, which is familiar to malignant cells and has been modified continuously to be increasingly tolerant to tumor growth and metastasis, the local microenvironment of a secondary organ can be much more unfriendly: different mechanical properties in extracellular matrices, different soluble factors and a relevantly intact local immune system [13], [29]. Such barriers lead to metastatic inefficiency, making organ colonization the most rate-limiting process of metastasis [13]: past research showed that more than half of tumor cells injected intravenously and metastasizing into lungs of immunodeficient mice were apoptotic 48 hours after tail vein injection [30]. Survival and proliferation pathways in metastatic cells can be inactivated spontaneously, can be suppressed by insufficient nutrients, or can be suppressed by immune cells, leading to dormancy that can last for years [31]. In such an adverse microenvironment, tumor cells that can sustain survival pathways relevant in the context of low levels of activating signals can persist [13]. It was found that primary tumors could release soluble factors and extracellular vesicles to secondary organs before arrival of metastatic cells, modifying local microenvironment, leading to formations of “pre-metastatic niches”, which were more favorable for survival and growth of metastatic cells [32].

Involvement of the Immune System in Cancer

Cancer cells constantly interact with their surrounding microenvironment, including ECM and non-cancer cells, during cancer development and metastasis [33]. Different cell types of the immune system

are recruited to cancer and interact with cancer cells during different stages of cancer development and metastasis [34], [35].

The immune system can distinguish cancer cells from normal cells by surface markers denoting cellular stress or abnormally upregulated in malignant cells. For example, phagocytic cells such as M1 macrophages can sense phosphatidylserines and calreticulin, two “eat-me” signals to induce phagocytosis, which were found to be upregulated in cancer cells compared to normal cells [36], [37]. Natural killer cells (NK cells), first identified as professional anti-cancer cells without deliberate activation [38], can recognize stress molecules absent in normal cells but highly expressed in multiple types of cancer, such as Hsp70, B7-H6 and MIC A/B, with NK activation receptors like NKp30 and NKG2D [39]-[41]. Neoantigens, which are mutated proteins presented by MHC I molecules, can induce cytotoxicity of CD8+ T cells, members of adaptive immunity [42]. Those cytotoxic T cells can also be stimulated by MHC-I-presented oncofetal and cancer-testis antigens, proteins that are normally expressed in fetuses or germ cells only [43], [44]. Furthermore, besides direct cytotoxicity, release of soluble factors by those cytotoxic cells themselves and other immune cell types such as CD4+ T cells and dendritic cells can strengthen cancer immunosurveillance by recruiting and activating more immune cells against transformed cells.

However, during cancer development, strategies of malignant cells to evade immunosurveillance are developed [45], [46]. Cancer cells expressing fewer antigens are gradually selected under immune stress [47]. Activation of specific oncogenes such as β-catenin and c-Myc and loss of specific tumor suppressors such as PTEN can inhibit activation or recruitment of innate immune cells such as macrophages and dendritic cells that can recruit and activate T cells [45]. Some immunosuppressive molecules are expressed by transformed cells to prevent immune infiltration, such as PGE2, which can inhibit infiltration of NK cells and recruitment of dendritic cells [48]. Tumor cells can also inhibit cytotoxic

immune cells by hijacking mechanisms relevant to self-tolerance and wound healing. For example, tumor cells can express ligands that can bind to immune checkpoints such as PD-1 and CTLA-4 on T cells to inhibit their activities [49], [50].

Tumor cells can also recruit or induce differentiation or conversion into “pro-tumor” immune cells that can inhibit anti-tumor ones and support cancer development and metastasis. For example, some types of cancer express IDO, which can both suppress cytotoxic immune cells such as CD8+ T cells and NK cells and generate immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressive cells (MDSCs) [51]-[53]. Secretion of chemokines and cytokines such as CCL2 by tumor cells leads to polarization of macrophages towards tumor-associated phenotypes [54]. Those pro-tumor immune cells can inhibit anti-tumor immune cells. Besides, tumor-associated macrophages (TAMs) can even promote tumor development, releasing factors supporting growth, angiogenesis, metastasis, and therapy resistance [55].

Involvement of the Immune System in Metastasis

Immune cells are also involved in metastasis [56]. Just like the case in primary tumors, CD8+ T cells and NK cells in secondary organs are mainly involved in immune surveillance against metastatic cells [57]. Interaction between local immune cells and metastatic cells cannot be decoupled from the primary tumor. On the one hand, immune response against a primary tumor is not limited locally but can induce concomitant immunity, which is anti-cancer immune response of the whole body, so that the possibility of secondary tumor occurrence is reduced [58]. On the other hand, factors released by primary tumors can polarize macrophages in secondary organs into pro-tumor phenotypes to help establish pre-tumor niches there [59]. Multiple pro-tumor immune cells such as neutrophils and MDSCs are shown to accumulate in pre-metastatic niches before arrival of metastatic cells [60], [61].

Dendritic Cells

Dendritic cells (DCs) are significant players in the immune system because they function as a bridge connecting the innate and adaptive immune system by antigen presentation [62], [63]. After derivation from hematopoietic bone marrow progenitor cells, immature DCs are distributed to peripheral tissues such as skin, gut, and lungs. There they sample local microenvironment by macropinocytosis of extracellular fluid [62]. They also phagocytose small pieces of membranes from live cells for sampling [64]. Those immature cells can also directly phagocytose pathogens like bacteria they encounter. As long as pathogens are detected by their pattern recognition receptors, they start maturation, downregulating molecules relevant to pathogen sampling and recognition [65], expressing molecules relevant to chemotaxis such as CCR7, antigen presentation such as MHC I and II, and T cell activation such as CD80, CD86 and CD40 [66]. DCs start their migration to lymph nodes via lymph vessels or the spleen via blood vessels [67], [68]. During migration, DCs digest engulfed molecules into peptides that can be presented with MHC I and II molecules recognizable for T cells, i.e., antigens. DCs also experience morphological changes, including formation of dendrites during this process, becoming really “dendritic”. After arrival to the spleen or lymph nodes, DCs can activate T cells recognizing presented antigens by binding between antigens on MHC molecules on DCs and TCRs on T cells, stimulated by ligand-receptor binding of costimulatory molecules like CD40, CD80 and CD86, and release of cytokines. DCs can activate CD4+ helper T cells through presentation via MHC II and CD8+ cytotoxic T cells through MHC I, in which the latter is called cross-presentation. In this way, adoptive immunity can be activated against presented antigens [62], [69].

DCs are heterogeneous according to lineage and resident tissues in both human and mice (reviewed in [70], [71]). Besides those derived directly from bone marrow progenitor cells, DCs can also be differentiated from monocytes under induction [72]. Generally, DCs are classified into conventional DCs

(cDCs) and plasmacytoid DCs (pDCs), and cDCs can be further divided into cDC1s and cDC2s. cDC1s are specialized in cross-presentation of antigens to cytotoxic CD8+ T cells, although they can also present antigens to CD4+ T cells [73]. cDC2s are more specialized in presentation to CD4+ T cells [74]. pDCs release IFN-γ, an interferon that is involved in activation of multiple immune cell types upon viral infection [75].

Dendritic Cells in Cancer

As antigen-presenting cells that can initiate adoptive immunity against presented antigens, DCs take an important role in anti-cancer immunity [76]. In Chen and Mellman's review [77], capture and presentation of neoantigens from malignant cells by DCs are considered as the first two steps in the cancer-immunity cycle, a cascade of immune responses after recognition of transformed cells, followed by infiltration of T cells in tumors. This model is further supplemented by observations in multiple solid tumors that DCs are recruited to tumors by NK cells that have infiltrated there [48], [78].

Among different subtypes of DCs, cDC1s are the most studied in their anti-cancer roles because of their abilities to directly activate cytotoxic CD8+ T cells by cross-presentation [48], [76]. However, other subtypes of DCs are also involved in cancer immunosurveillance directly or indirectly [79]. Injection of pDCs activated with tumor antigen-associated peptides into metastatic melanoma patients was found to induce anti-tumor T cell responses [80]. cDC2 can recruit CD4+ helper T cells, which have been shown to be required to maintain optimal cytotoxicity and prevent exhaustion of CD8+ T cells [81], [82]. Therefore, multiple subtypes of dendritic cells should be considered to explain DCs' involvement in cancer immune surveillance.

Tumors can prevent infiltration of immune cells by suppress activities of DCs. For example, downregulation of CCL4 induced by Wnt/β-catenin signaling was found to suppress infiltration of DCs

[83]. PGE2 was reported to suppress recruitment and activities of cDC1 to prevent infiltration of T cells [48]. DCs can be induced to be dysfunctional and immunosuppressive by malignant cells [84]. In an ovarian mouse model, DCs infiltrating in tumors were found to be transformed from immunostimulatory to immunosuppressive during cancer development because of PGE2 and TGF-β1 in tumor microenvironment (TME) [85]. Dysfunctional pDCs were reported to recruit Tregs and MDSCs to tumors and inhibit activities of cytotoxic T cells, and depletion of pDCs was found to reduce breast cancer bone metastases [86].

Compared with that on primary tumors, there is much less research on involvement of DCs in secondary organs seeded by metastases. In one paper covering this topic, DCs of an immunosuppressive subtype differentiated from monocytes were found to accumulate surround early pancreatic cancer liver metastases, which could recruit Tregs and inhibit CD8+ T cells [87]. Therefore, it requires further investigation whether DCs localized in secondary organs are involved in interaction with metastatic cells, especially anti-metastasis responses.

Identification of Dendritic Cells

Identification and discrimination of different immune cell types and subtypes is challenging in peripheral tissues, such as tumors. Dendritic cells are heterogeneous and no exception. CD11c (Integrin alpha X) is a widely used dendritic cell marker in experiments with mice [88][89]. Some research uses CD11c as a definitive DC marker [90]. However, though once thought to be exclusive to DCs [91], CD11c can be expressed in other immune cells, such as macrophages [92]-[94]. Although in most of those cell types, CD11c is expressed at low level or transiently, macrophages resident in several organs, such as lungs and intestines, were found to express a similar level of CD11c to DCs during steady-state condition, according to flow cytometry [95]. Therefore, another marker is necessary for classification of dendritic cells and macrophages in mouse lungs. CD24, which is expressed in dendritic cells but not in

macrophages, has been used in this situation to distinguish the two myeloid cells in flow cytometry of several experiments [95]-[97]. In conclusion, additional staining of CD24 may improve the accuracy of DC labeling in immunofluorescence as in flow cytometry.

Extracellular Vesicles

Extracellular vesicles (EVs) are lipid-bilayer particles released by multiple types of cells. Containing various proteins, micro-RNAs, lipids, and other molecules, EVs can be used for cell-cell distant communication besides direct release of soluble factors or removal of waste from cells (reviewed in [98]). Generally, EVs released from normal cells are classified into exosomes and microvesicles (MVs, or ectosomes) according to size and mechanisms of genesis. Exosomes originate from endosomes formed by inward budding of the cellular membrane. These endosomes become multivesicular bodies (MVB) by accumulation of many smaller vesicles inside by further inward budding. Those smaller vesicles, containing cellular contents, are released from cells by fusion of MVBs with the cellular membrane, becoming exosomes [99]. MVs are directly released by outward blebbing of the cellular membrane, resulting from changes of lipid and protein components and Ca^{2+} levels [100]. The two MVs are mainly distinguished by their sizes: while the diameter of an exosome range from 30 to 150 nm, that of an MV range from 100 to 1000 nm [100]. They can also be labeled by their specific surface markers, such as CD9, CD63, and CD81 for exosomes and integrins and ARF6 for MVs [101], [102]. Besides, during apoptosis, dying cells release apoptotic bodies, 50-5000nm in size, containing anything in those cells, including fragments of nuclei [103]. As carriers of information between cells, EVs are involved in various physiological and pathological activities, including immune responses.

Extracellular Vesicles in Cancer

Multiple types of EVs have been reported to be released by cells in tumor, both malignant and non-malignant ones. Those EVs take important roles in immunity against cancer, cancer development, and metastasis [104], [105].

During the whole process of cancer development, cancer cells release both exosomes and MVs, the latter of which are also called oncosomes and can be as large as 10 μm in diameter [106]. Those EVs can be absorbed by other malignant cells, so that oncogenic molecules can be shared by different tumor subpopulations [107]. EVs from tumor cells are also released to non-tumor cells in TME. Tumor EVs transferred to fibroblasts and endothelial cells were reported to facilitate metastasis by promoting ECM remodeling and angiogenesis [108], [109]. Those particles released by malignant cells are also received by immune cells, leading to mixed responses of the immune system. EVs containing MHC I and tumor antigens can induce immune responses by DCs and T cells against cancer [110], [111]. However, those tumor-cell-released EVs can promote immune evasion through modulation of immune cells by contents such as microRNAs and cytokines inside particles, including induction of apoptosis by immune cells, suppression of cytotoxic activities, recruitment of immunosuppressive immune cells, and induction of immunosuppressive phenotypes [112]-[115]. Tumor EVs can also enhance tumor-supporting activities of pro-tumor immune cells, such as angiogenesis [112]. During metastasis, EVs released from primary tumors can promote formation of pre-metastatic niches by increasing vascular leakiness, inducing angiogenesis, and recruiting pro-tumor cells or converting local-tissue resident cells [116]-[119].

Non-tumor cells in TME also transfer EVs to surrounding tumor cells or non-tumor cells. Exosomes transferred from fibroblasts to cancer cells can promote tumor invasion and therapy resistance [120]. EVs secreted by immune cells can have a mixed influence on cancer development. On the one hand, macrophages can transfer exosomes with cancer antigens to dendritic cells [121]; dendritic cells can

implement antigen presentation via EVs to activate CD4+ and CD8+ T cells [122]; both can initiate anti-cancer immune responses. On the other hand, EVs released by immunosuppressive cells such as Tregs can be transferred to anti-tumor immune cells to inhibit their activities [123]. Furthermore, EVs released by MDSCs were reported to promote breast cancer lung metastasis by inducing Th2 cells and angiogenesis [124]. However, direct transfer of EVs or even cytoplasmic contents from immune cells to cancer cells has been much more rarely reported, although it was reported that cytoplasmic contents of macrophages were directly transferred to melanoma via cell-cell contact, which enhanced dissemination of recipient malignant cells [125]. Therefore, investigation of transfer of EVs from immune cells to cancer cells is valuable.

Dendritic Cells and Extracellular Vesicles

DCs are one of the first cell types to be reported to secrete exosomes [122]. EVs with different sizes and markers have been found to be secreted by DCs. [102] Contents of EVs and relative proportions of exosomes and MVs are also different between immature and mature DCs [102], [126]. The EVs containing MHC I, MHC II or both can perform antigen presentation to T cells in place of or along with DCs in multiple ways: those EVs can be released by DCs in peripheral tissue and migrate to lymph nodes to activate T cells directly or indirectly after being recruited by bystander DCs in lymph nodes; they may also be still attached to DCs after secretion and presented to T cells after migration with associated DCs; DCs may form MVBs after being activated by pathogens and release vesicles inside MVBs as exosomes during antigen-presentation to T cells [127]. Besides MHC molecules, cytokines like IL-1β were also found to be contained in DC-released EVs [128]. However, exosomes derived from DCs treated with IL-10 or TGF-β1 were reported to have immune suppression functions [129], [130].

When they were first discovered in tumor-bearing mice, exosomes derived from DCs were already found to activate anti-cancer cytotoxic CD8+ T cells, and they could be used to eradicate tumors as vaccines

[122]. Those DC-derived exosomes can also activate NK cells with ligands to bind to multiple NK activation receptors such as TNFR, NKG2D, IL-15Ra, and NKp30 [131]-[133]. However, considering that TGF-β1 has been reported to induce phenotypical changes of DCs infiltrating to ovarian cancer, it is reasonable to deduce that their EVs are also immunosuppressive [85], [129].

About This Experiment

In the experiment, we sought to investigate the interaction between CD11c+CD24+ dendritic cells resident in lungs and metastatic breast cancer cells during colonization of metastases in lungs. We injected mammary tumor clusters isolated from PyMT-MMTV mouse models into wildtype FVB mice through their tail veins. The PyMT-MMTV model is established through insertion of the gene of polyomavirus middle T antigen fused (PyMT) following long terminal repeats of the mouse mammary tumor virus (MMTV). The transgenically expressed PyMT oncogene transforms mammary epithelial cells because of activation of tyrosine kinase activities of c-Src families, leading to formation of tumors similar to luminal B breast cancer [134]. PyMT-MMTV mice develop lung metastases within 3 months, making them the most common genetically engineered mouse model in breast cancer metastasis research.

Lungs of injected mice were dissected at different time points and processed into tissue sections. The potential interaction between DCs and metastases was investigated by identification, quantification and characterization of DC contents in metastases and DCs surrounding metastases through immunofluorescence of tissue sections. We cannot draw a definite conclusion about whether lung dendritic cells are involved in immune responses against infiltrating metastatic cells. We conclude by identifying the next steps in research that would be required to definitively investigate this interaction.

Results

CD11c+ Particles are Found in Lung Metastases Formed by Intravenously Injected PyMT-MMTV Mammary Tumor Cells

In the experiment, we dissected mammary tumors from mice of the ROSAmT/mG;PyMT-MMTV model, in whose cells express tdTomato fluorescence proteins [19]. We then isolated tdTomato+ tumor clusters by mechanically mincing and enzymatically digesting dissected tumors into organoids and trypsinizing organoids into single cells or clusters with 2-10 cells, which are both mentioned as "clusters" hereinafter. Then about one million clusters were injected into the tail vein of an unlabeled wildtype FVB mouse. Lungs were dissected and fixed from the injected mouse 2 h after tail vein injection. We then sectioned the lungs into slides and stained one slide with an antibody against CD11c, the classical marker of dendritic cells. The schema of the experiment is shown in Figure 1.

We found CD11c+ particles colocalized to lung metastases expressing tdTomato (Figure 2A, C, D, F). Those CD11c+ particles were found overlapped with 158 of 179 metastases on the lung section (Figure 2H). Each metastasis was overlapped with about 7 CD11c+ particles on average (Figure 2G), although the distribution of those particles was highly diverse, with examples overlapping with no or many CD11c+ particles found. Large metastases with many particles are shown in Figure 2A and Figure 2C; a large metastasis without any particle is shown in Figure 2E; three small metastases with no, one and seven particles respectively are shown in Figure 2D. Furthermore, some metastases were wholly or partially stained with the anti-CD11c antibody besides colocalized with CD11c+ particles. Such cell- or cluster-level staining usually showed lower fluorescence intensity. (Figure 2F). According to Figure 2E, sizes of CD11c+ particles were highly various, ranging from 0.5 to 6 μm in diameter, with the average 1.33 μm. Figure 2F shows that about 36% of those particles are smaller than 1 μm in diameter and thus fulfill the size criteria of ectosomes [98], and the rest 64% are larger than 1 μm.

In order to distinguish whether the overlapping particles were inside metastatic cells or on their cell surfaces, another slide was stained without permeabilization and use of Triton X-100. Because tissue fixing with paraformaldehyde (PFA) in PBS solution cannot permeabilize cellular membranes thoroughly, permeabilization with detergents like Triton X-100 is required for staining intracellular molecules [135]. In other words, omission of permeabilization and removal of Triton X-100 in each buffer used in tissue immunofluorescence staining can lead to labeling of membranous proteins with a greatly reduced possibility of staining inside the cells [136], [137]. We refer to staining with permeabilization as "complete staining" and staining without Triton X-100 as “membranous staining”.

Figure 2H and 2G show that membranous staining successfully removed CD11c+ particles seen to overlap with metastases on the complete staining slide: only 28 of 173 metastases are found to be overlapped with CD11c+ particles. At the same time, the staining of CD11c on cellular membranes was maintained (Figure 2C). Accordingly, our data suggest that these CD11c+ particles are mostly located inside metastatic cells.

Co-staining with CD11c and CD24 Antibodies Shows that the CD11c+ Particles May Not Come from Dendritic Cells

Although CD11c is widely used as a classical marker of DCs, this molecule is also found in other cell types. We tried to further characterize the CD11c+ particles in the metastases to see whether they derived from DCs. Since in mouse lungs, macrophages express CD11c at a similar level to dendritic cells [95], we stained CD24, a marker to distinguish dendritic cells and macrophages, along with CD11c in another section to verify whether the CD11c+ particles found were derived from dendritic cells. We assumed that particles derived from CD11c+ CD24+ dendritic cells could be stained with both antibodies.

Figure 3A shows how CD11c+ and CD11c+ CD24+ are identified: CD11c+ particles are particles with higher fluorescence intensity compared to its surroundings; CD11c+ CD24+ particles are determined by

checking whether a CD24+ particle, with higher fluorescence intensity compared to its surroundings, colocalize with a detected CD11c+ particle. Although cell- or metastasis-level staining of CD11c and CD24 was found in about 36% and 27% of metastases we analyzed respectively, CD11c+ and CD24+ particles with higher fluorescence intensity are still identifiable (examples shown in Figures 3A and E). If a CD24+ particle overlap partially with a CD11c+ particle, they are not considered as a CD11c+ CD24+ particle, as explained in Figure 3E.

We found that most CD11c+ particles were not co-stained with CD24: among 437 metastases analyzed on this section, the mean of CD11c+ particles (including CD11c+ CD24+ particles) in a metastasis was 1.2, while that of CD11c+ CD24+ particles was 0.06 only; about 41% were detected with CD11c+ CD24- particles , while only 1.83% were with CD11c+ CD24+ particles (Figure 3C). An example of metastases with many CD11c+ particles but no co-stained particles is shown in Figure 3E. Those results show that most of those CD11c+ particles do not co-stain with CD24.

Particle Numbers in a Metastasis is Found to Be Correlated With its Cell Number

We analyzed whether the number of particles in a metastasis was relevant to its size, represented by the cell number in a metastasis. We classified metastases co-stained with antibodies against CD11c and CD24 into three groups: 136 individual cells, 259 small clusters with 2-10 cells, and 43 large clusters with more than 10 cells. While 80% of individual cells were found without any particle, the percentage was reduced to 54.83% for small clusters and lower than 20% for large clusters; in contrast, about 60% large clusters were colocalized with more than one particle, while that percentage was only about 6% for individual cells (Figure 3F). It shows that the distribution of particles among metastases was correlated with the size of a metastasis.

Characterization of CD11c+ Particles Through Staining of Arf6

We tried to determine the type of CD11c+ particles. Since the particles were too large to be exosomes, whose sizes should be 50-150 nm in diameter, and some of them fell under the size range of ectosomes, we stained another slide with antibodies against CD11c and Arf6. Multiple membrane proteins have been labeled as markers of ectosomes [138]. We chose Arf6 among those markers in the experiment because Arf6 is specifically involved in release of ectosomes [101]. While other ectosomemarkers, such as EGFR and integrins, may also be expressed in other vesicles released by cells such as apoptotic bodies.

The procedures of determination of CD11c+ Arf6+ particles are similar to those of determination of CD11c+ CD24+ particles: they are determined by checking whether an Arf6+ particle, with higher fluorescence intensity compared to its surroundings, colocalize with a detected CD11c+ particle. Figure 4A shows a metastasis expressing CD11c+ Arf6+ particles.

However, many fewer metastases were found to be overlapped with CD11c+ particles on this slide, not mentioning CD11c+ Arf6+ particles. Among 88 metastases analyzed, only 12.6% were found with particles with CD11c staining and 3.4% were found with CD11c+ Arf6+ particles (Figure 4D). In other words, among 23 CD11c+ particles found on the slide, 4 were co-stained with two antibodies (Figure 4E).

Furthermore, more than half of the metastases were found to be stained with the Arf6 antibody thoroughly: while for metastases without CD11c+ particles, half were stained with Arf6 (as shown in Figure 4B), and half were not stained (as shown in Figure 4C), all metastases with CD11c+ particles were stained like Figure 4A (Figure 4F).

Quantification of CD11c+ and CD11c+ CD24+ Cells Surrounding Metastases

We then investigated dendritic cells surrounding metastases by assessing membranous staining of antibodies against CD11c and CD24. We first counted CD11c+ cells located within a circle with a radius of 100 μm with a metastasis as its center, and then checked whether their membranes were also stained

with CD24 at least partially. An example is shown in Figure 5A. Some cells were seen to overlap with blots of CD24 staining, but shapes of those cells and blots did not completely overlap. In such cases, those blots were not considered as results of expression of CD24 on those cells, and those cells were not counted as CD11c+ CD24+ cells.

Figure 5C shows that there were 5.88 cells expressing CD11c surrounding a metastasis on average, and among them 4 also expressing CD24 and were thus considered as dendritic cells. It shows that staining of CD24 besides CD11c can filter other CD11c-expressing cells from dendritic cells.

As shown in Figure 5B, metastases were various in size: among 138 metastases analyzed, 10.14% were individual cells, 75.36% were small clusters with 2-10 cells, and 14.50% were large clusters with more than 10 cells. We attempted to investigate the potential relationships between the size of a metastasis and numbers of cells expressing CD11c (including co-stained cells) or expressing both CD11c and CD24 surrounding it. According to Figure 5D (for CD11c+ cells) or Figure 5E (for co-stained cells), we did not find a correlation between metastasis size and numbers of surrounding stained cells.

Metastases were not distributed homogeneously, with some gathering in some regions. Therefore, we also investigated whether metastasis density led to strengthened gathering of antigen-presenting cells surrounding metastases, especially if two metastases were located within 100 μm. We classified metastases into those distributed in “low density”, whose distances from other metastases were at least 100 μm, as shown in Figure 5F, and those distributed in “high density”, which were nearer than 100 μm away from at least one another metastasis, as shown in Figure 5G. The comparison of CD11c+ cells or CD11c+ CD24+ cells surrounding low-density and high-density metastases does not show correlation between accumulation of metastases and of stained cells (Figure 5H).

Membranous Staining Removes Cell- or Metastasis-Level Staining of CD11c but Not Staining of CD24

We investigated that whether membranous staining without Triton-X-100-mediated permeabilization influenced cell- or metastasis-level staining of metastases by two antibodies, since metastases stained with the CD11c antibody, the CD24 one or both were found in both sections, as shown in Figures 3A, 3E and 5A. According to Figure 3D, among 448 metastases analyzed on the permeabilized slide, about 36% were thoroughly or partially stained with the CD11c antibody (examples can be seen in Figures 3A and 3E), while only 8% of 138 metastases on the slide stained without Triton X-100 were stained that antibody. On the contrary, 119 metastases stained completely, and 78 metastases stained membranously were found to be stained with the CD24 antibody. It indicates that membranous staining can remove cell- or metastasis-level staining of the CD11c antibody but not that of the CD24 antibody.

Dynamics of Dendritic Cells Between 6 h and 7 d After Tail Vein Injection

In order to understand how dendritic cells are involved in early time of colonization of metastasis, we injected 250,000 tdTomato+ PyMT-MMTV clusters into each of four wildtype FVB mice, and dissected their lungs 6 h, 24 h, 48 h, and 7 d after tail vein injection respectively. Then those lungs were sectioned into slides for further use. First, we determined the densities of metastases of those lungs. Examples of those sections are shown in Figure 6A. Based on two serial sections, metastasis density kept decreasing during the duration from 0.8 metastasis per cm^2 6 h after injection to fewer than 0.1 metastasis per cm^2 7 d after injection (Fig. 6B). The 7-d sample was not used later because of too low metastasis density.

Then 2 sections were selected from each time point and were stained with antibodies against CD11c and CD24 membranously. Then the numbers of CD11c+ and CD11c+ CD24+ cells were counted through the method mentioned in Figure 5A. As shown in Figure 5C, 43 metastases, with 25 individual cells and 18 clusters were analyzed on 6 h slides; 30 metastases, with 9 individual cells and 21 clusters were analyzed on 24 h slides; 3 single cells were analyzed on 48 slides. Figure 5D shows that there is no significant change in numbers of CD11c+ cells or CD11c+ CD24+ cells surrounding metastases between 24 h and 48

h: there were 3.34 cells expressing CD11c surrounding a metastasis on average, in which 2.41 cells also expressed CD24; as for 48-h slides, the average CD11c+ cell number was 3.1 while that of CD11c+ CD24+ cells was 2.5. The 48-h metastases were not considered because they were too few. A 24-h example is shown in Figure 5E and a 48-h example is shown in Figure 5F.

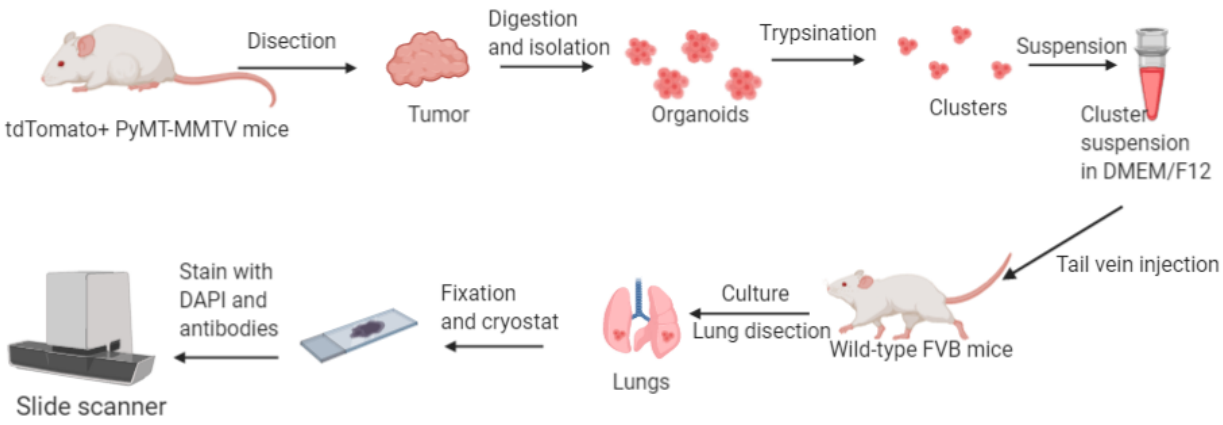
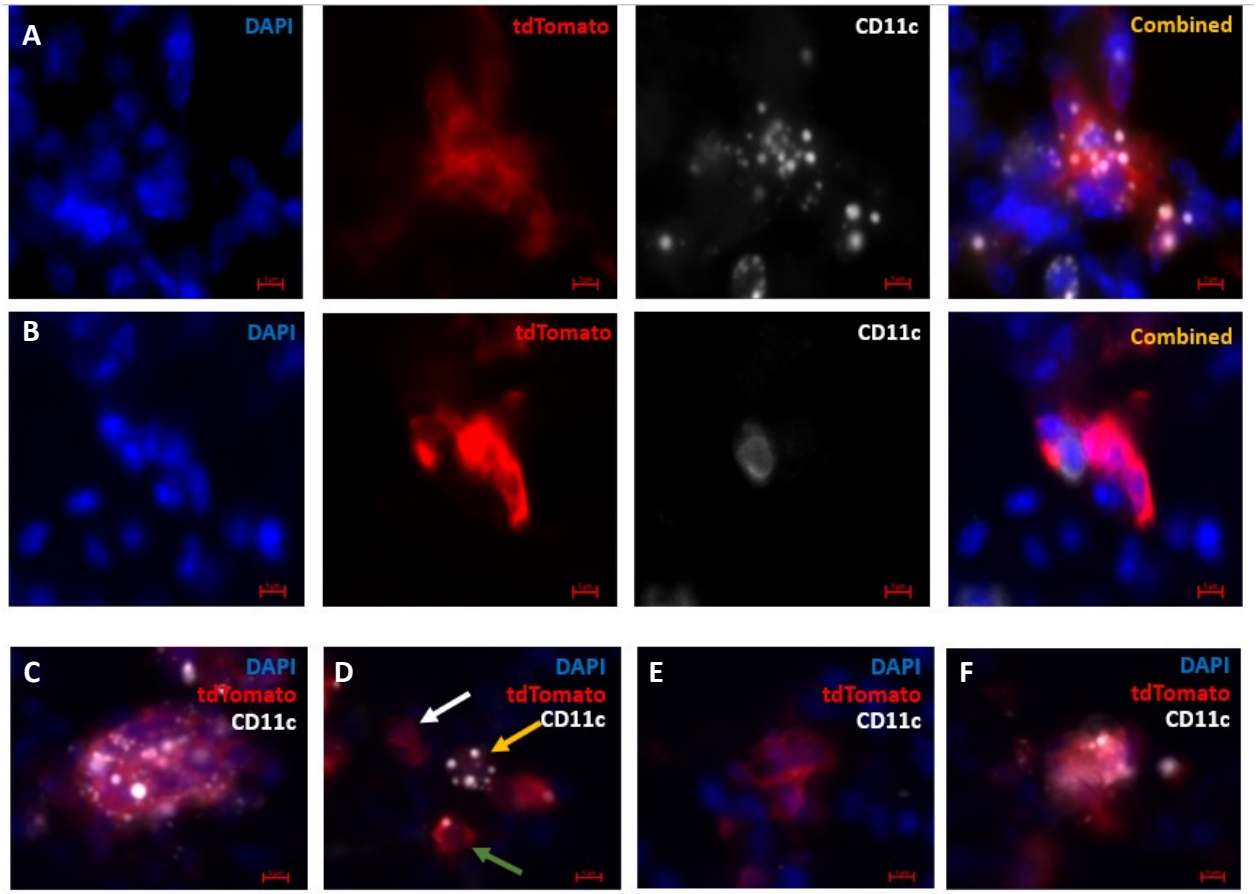
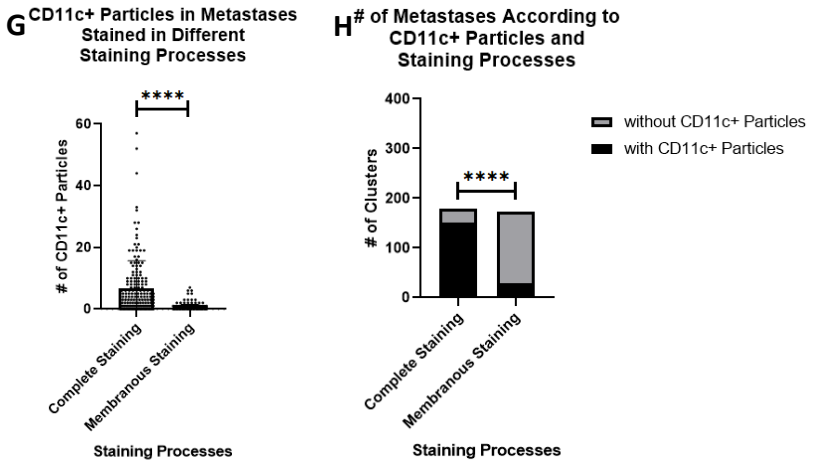


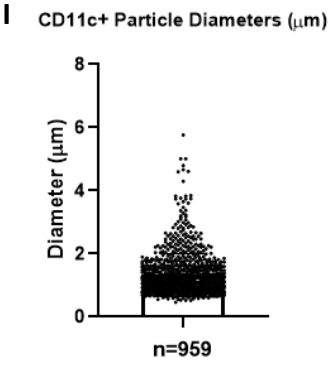
Figure 1 Schematic of Lung Metastasis Assay

Clusters that are isolated from tumors of PyMT-MMTV mice expressing tdTomato markers are injected into wildtype mice, whose lungs are dissected after a designated time. The dissected lungs are then fixed and sliced into sections, which are stained with DAPI and designated antibodies. Created with Biorender.


A
DAPI
tdTomato
CD11c
Combined
B
DAPI
tdTomato
CD11c
Combined
C
DAPI
tdTomato
CD11c
D
DAPI
tdTomato
CD11c
E
DAPI
tdTomato
CD11c
F
DAPI
tdTomato
CD11c


G
CD11c+ Particles in Metastases Stained in Different Staining Processes
****
# of CD11c+ Particles
60
40
20
0
Complete Staining
Membranous Staining
Staining Processes

H
# of Metastases According to CD11c+ Particles and Staining Processes
without CD11c+ Particles
with CD11c+ Particles
****
# of Clusters
400
300
200
100
0
Complete Staining
Membranous Staining
Staining Processes


I
CD11c+ Particle Diameters (µm)
Diameter (µm)
8
6
4
2
0
n=959

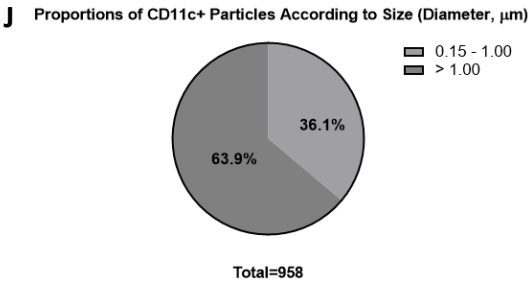

J
Proportions of CD11c+ Particles According to Size (Diameter, µm)
0.15 - 1.00
> 1.00
36.1%
63.9%
Total=958

Figure 2 CD11c+ Particles are Found in Lung Metastases

(A-B) Comparison between metastases stained through complete staining (A) or membranous staining (B).

(C-E) Different CD11c+ staining situations shown in metastases:

(C) A large metastasis with many CD11c+ particles.

(D) Three small metastases with multiple particles (pointed by the yellow arrow), one particle (green arrow) or no particle (white arrow) respectively.

(E) A large metastasis with no CD11c+ particle.

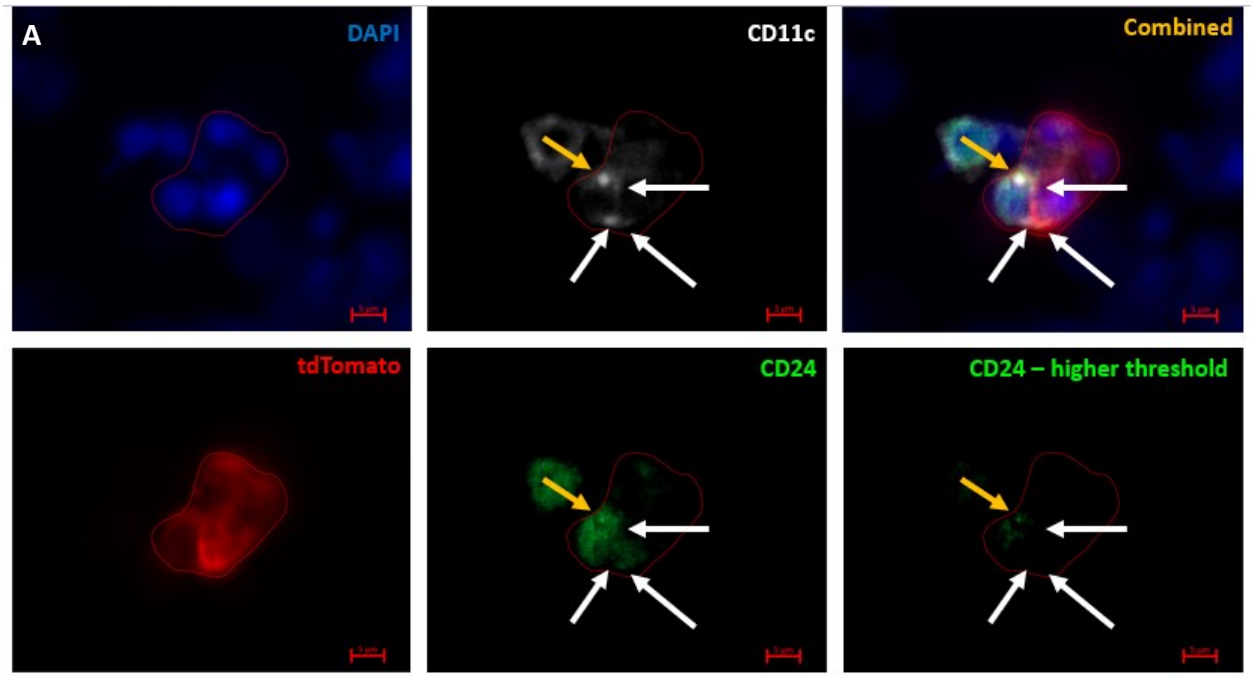
(F) A large metastasis with both CD11c+ particles and a metastasis-level CD11c+ staining.

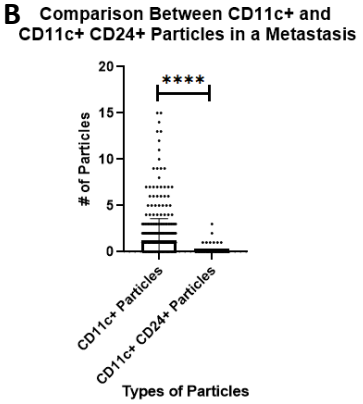
(G) Numbers of particles found in tdTomato+ metastases on sections stained through different staining processes. (Data are presented as mean ± SD, Kolmogorov-Smirnov test, $p<0.0001$).

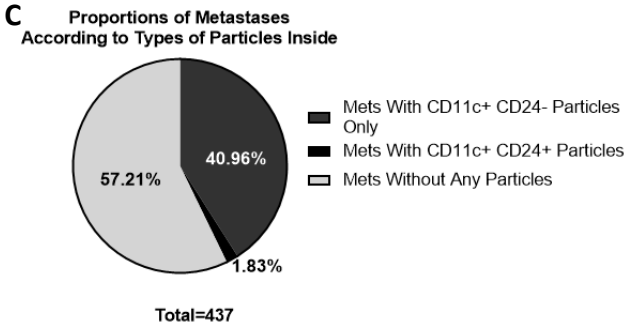
(H) Proportions of metastases stained with or without tdTomato+ on sections stained through different staining processes. (Fisher's exact test, two-sided, $p<0.0001$).

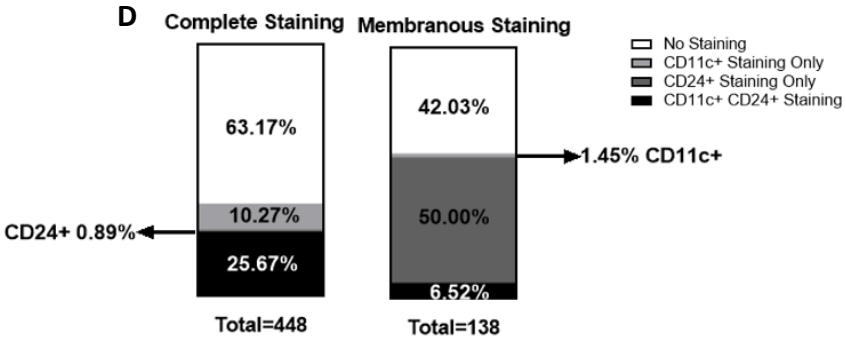
(I) Sizes of CD11c+ particles.

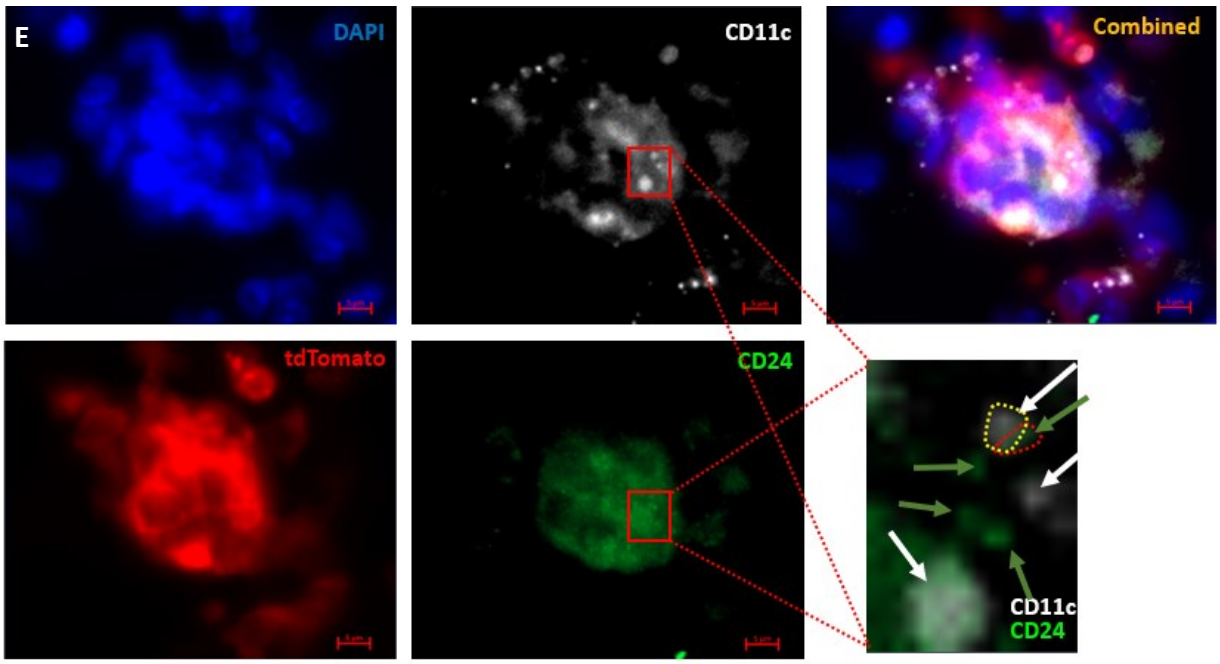
(J) Proportions of CD11c+ particles according to size.


A
DAPI
CD11c
Combined
tdTomato
CD24
CD24 – higher threshold


B Comparison Between CD11c+ and CD11c+ CD24+ Particles in a Metastasis
****
# of Particles
20
15
10
5
0
CD11c+ Particles
CD11c+ CD24+ Particles
Types of Particles


C Proportions of Metastases According to Types of Particles Inside
40.96%
57.21%
1.83%
Mets With CD11c+ CD24- Particles Only
Mets With CD11c+ CD24+ Particles
Mets Without Any Particles
Total=437


D
Complete Staining
Membranous Staining
No Staining
CD11c+ Staining Only
CD24+ Staining Only
CD11c+ CD24+ Staining
63.17%
10.27%
25.67%
CD24+ 0.89%
42.03%
1.45% CD11c+
50.00%
6.52%
Total=448
Total=138


E
DAPI
CD11c
Combined
tdTomato
CD24
CD11c
CD24

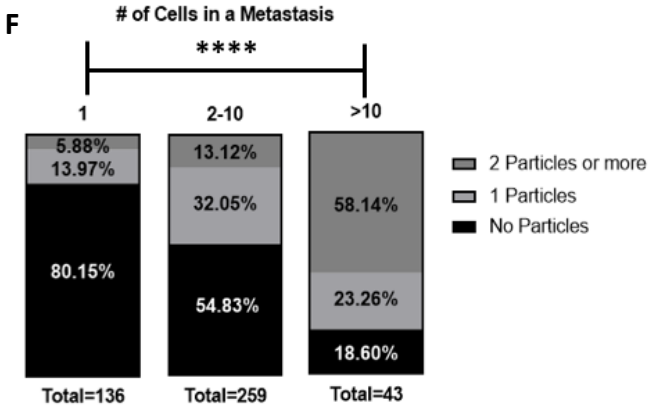

F
# of Cells in a Metastasis
****
1
2-10
>10
5.88%
13.97%
80.15%
13.12%
32.05%
54.83%
58.14%
23.26%
18.60%
Total=136
Total=259
Total=43
2 Particles or more
1 Particles
No Particles

Figure 3 CD11c and CD24 Double Staining to Determine the Source of CD11c+ Particles

(A) Determination of CD11c+ and CD11c+ CD24+ particles: CD11c+ particles are firstly found according to the AF647 channel (CD11c) image, a CD11c+ particle should be a particle with much higher AF647 intensity than its surroundings, even if the its surroundings are also stained, then the AF488 (CD24) channel image is checked to see whether the location of the CD11c+ particle is also stained with CD24 with much higher intensity. In this case, the particle pointed by the yellow arrow is considered CD11c+ CD24+, while ones pointed by white arrows are not, because they are not stained with CD24, or the staining is not brighter than its surrounding.

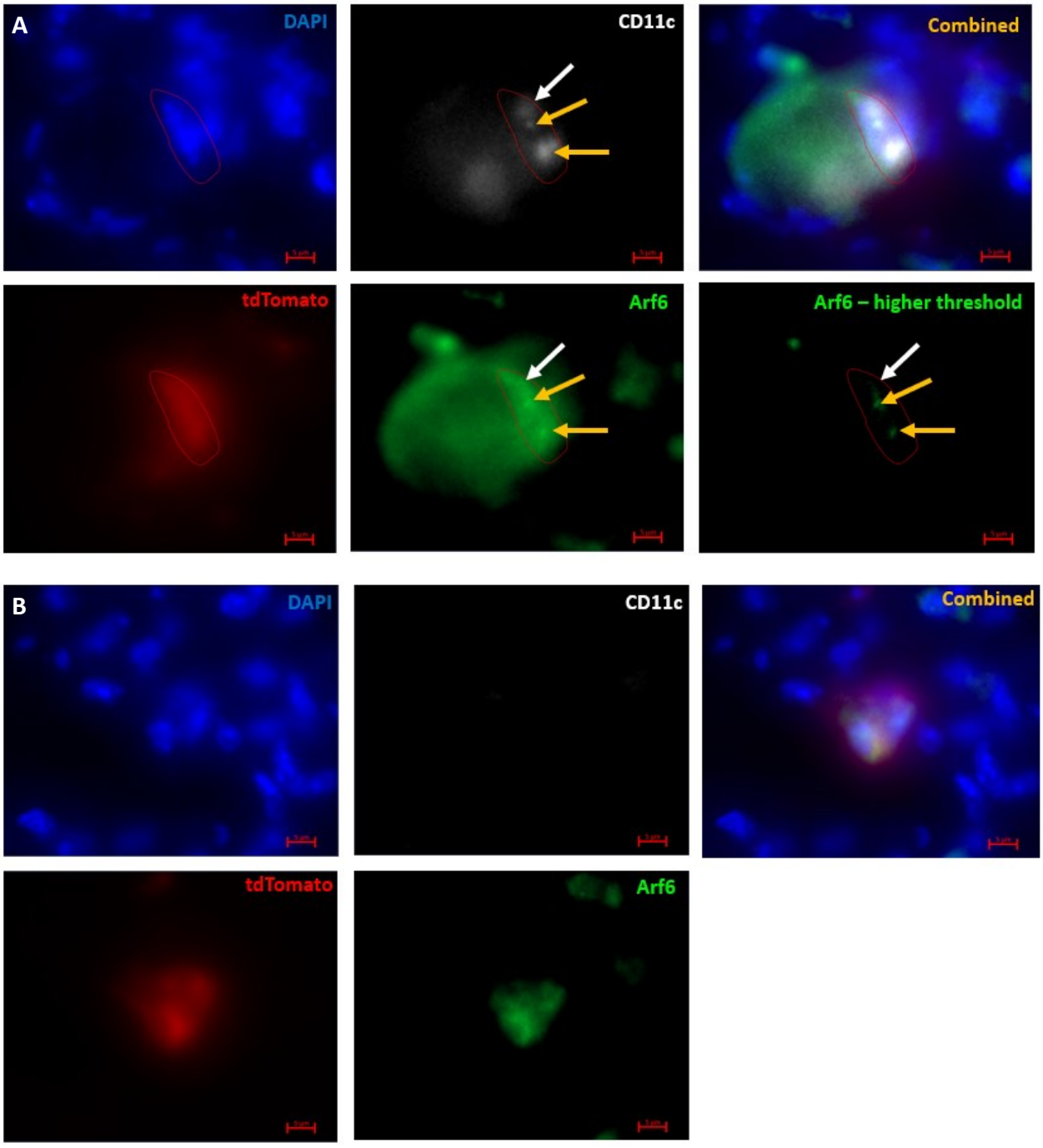
(B) Numbers of CD11c+ and CD11c+ CD24+ particles in a metastasis (CD11c+ CD24+ particles are included in CD11c+ particles. Data are presented as mean ± SD, Kolmogorov-Smirnov test, $p<0.0001$).

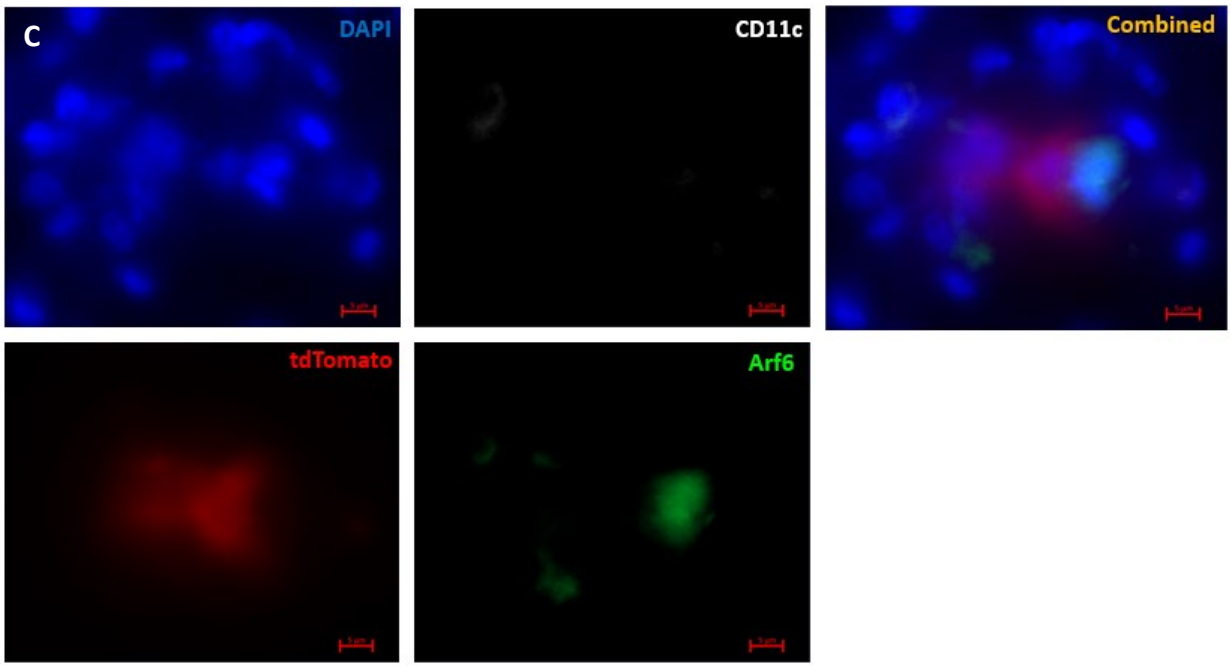
(C) Proportions of metastases according to types of particles inside.

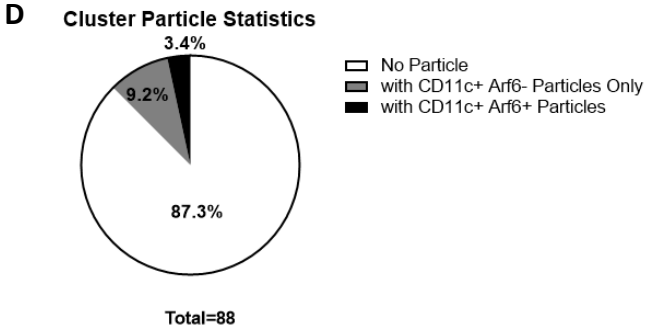
(D) Proportions of Metastases on sections through complete or membranous staining according to cell- or metastasis-level staining of CD11c or CD24 antibodies.

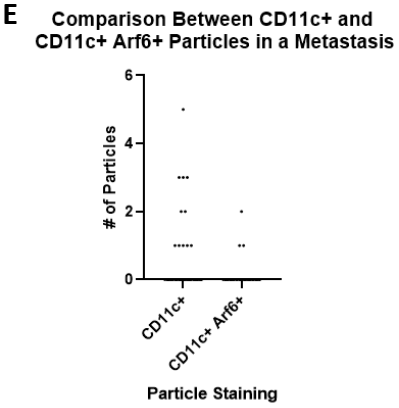
(E) An example of a metastasis which is stained with both CD11c and CD24 in a metastasis level and is found with quite a few CD11c+ particles but no CD11c+CD24+ particles (A region containing three CD11c+ particles is enlarged, with the thresholds of CD11c and CD24 modified to highlight particles. CD11c+ particles pointed by white arrows do not overlap with CD24 particles pointed by green arrows. One CD11c+ particle is circled with yellow dots to show that it does not overlap with the CD24 particle circles with red dots.

(F) Percentages of metastases according to CD11c+ particles inside, divided into three groups according to cell numbers of metastases: individual cell, small clusters with 2-10 cells, and larger clusters with more than 10 cells (Chi-square test, $p<0.0001$).


A
DAPI
CD11c
Combined
tdTomato
Arf6
Arf6 – higher threshold
B
DAPI
CD11c
Combined
tdTomato
Arf6


C
DAPI
CD11c
Combined
tdTomato
Arf6


D
Cluster Particle Statistics
3.4%
9.2%
87.3%
No Particle
with CD11c+ Arf6- Particles Only
with CD11c+ Arf6+ Particles
Total=88


E
Comparison Between CD11c+ and CD11c+ Arf6+ Particles in a Metastasis
# of Particles
0
2
4
6
CD11c+
CD11c+ Arf6+
Particle Staining

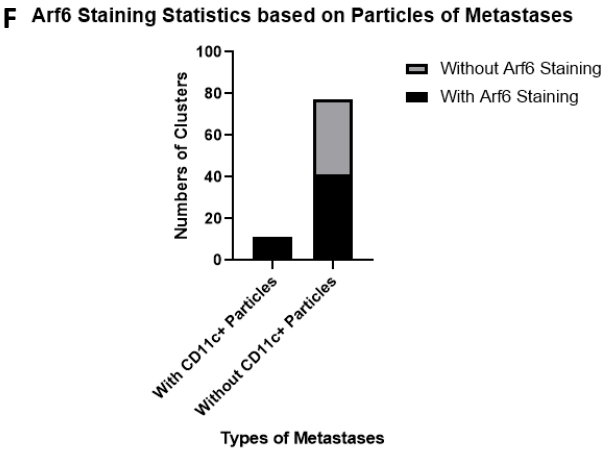

F
Arf6 Staining Statistics based on Particles of Metastases
Numbers of Clusters
0
20
40
60
80
100
Without Arf6 Staining
With Arf6 Staining
With CD11c+ Particles
Without CD11c+ Particles
Types of Metastases

Figure 4 Investigation of the Type of CD11c+ Particles with Arf6, an Ectosome Marker

(A) Determination of CD11c+ and CD11c+ Arf6+ particles: similar to determination of CD11c+ and CD11c+ CD24+ particles. One CD11c+ particle is pointed by a white arrow, and two CD11c+ particles are pointed by yellow arrows.

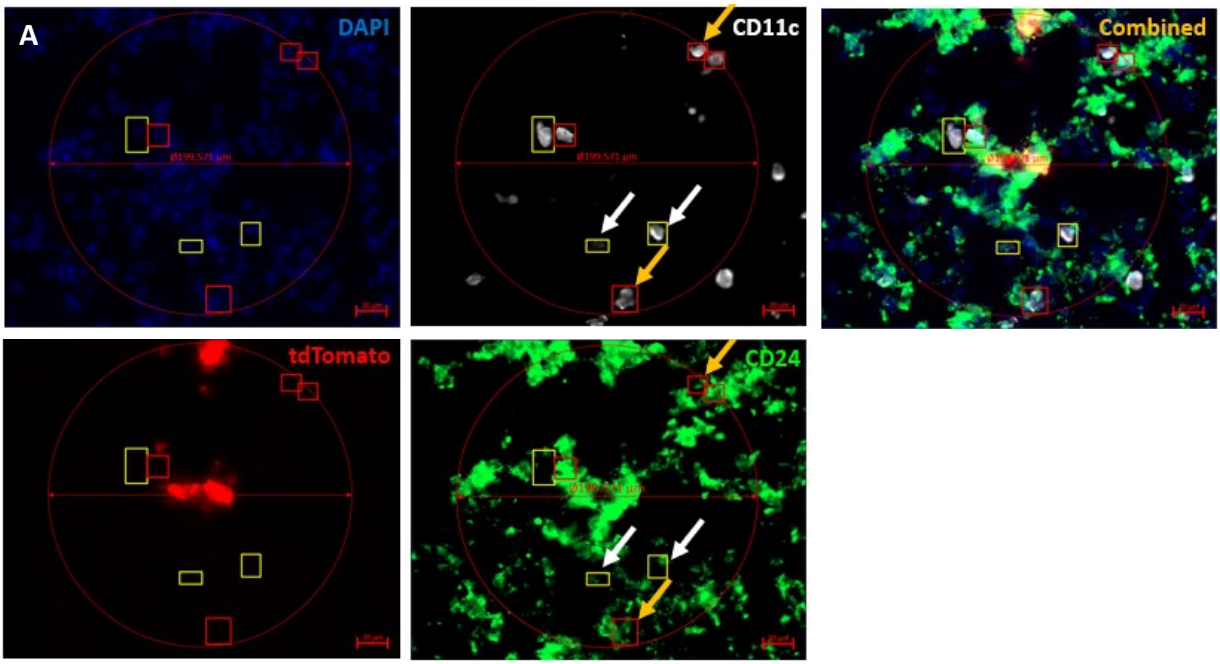
(B) An example of metastases without CD11c+ particles and are stained with Arf6.

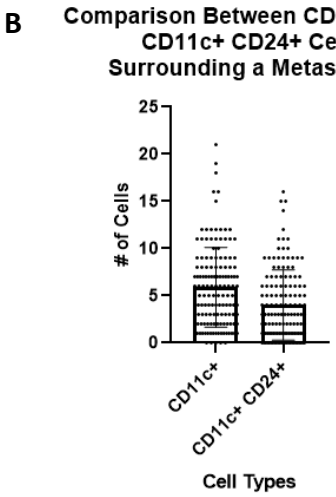
(C) An example of metastases without CD11c+ particles and are not stained with Arf6.

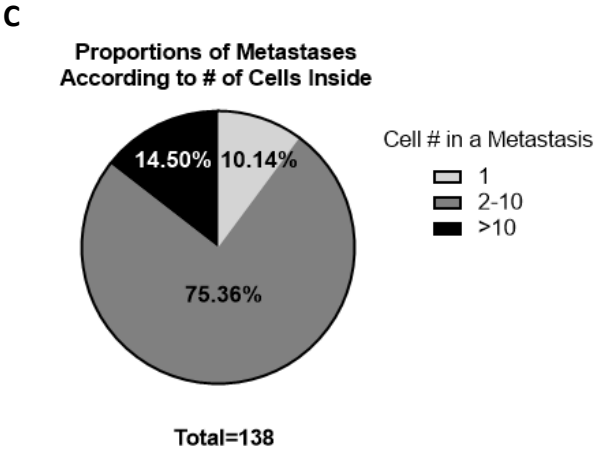
(D) Proportions of metastases according to types of particles inside.

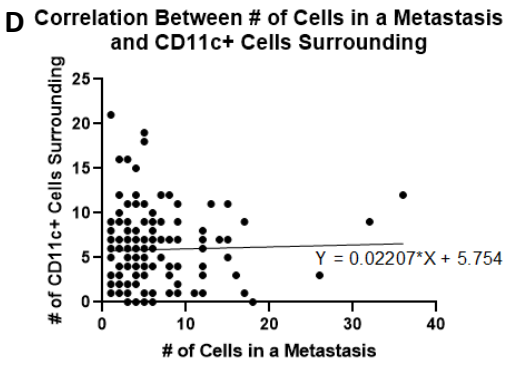
(E) Numbers of CD11c+ and CD11c+ Arf6+ particles in a metastasis (CD11c+ Arf6+ particles are included in CD11c+ particles).

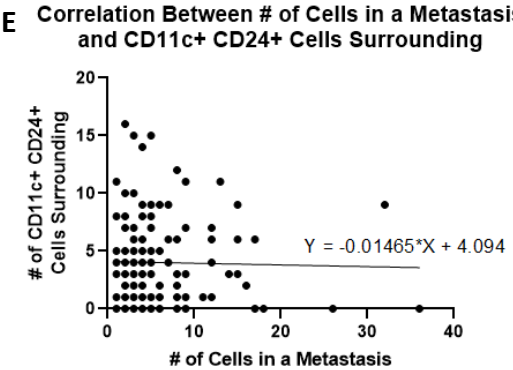
(F) Comparison of Arf6 staining between metastases with or without CD11c+ particles.

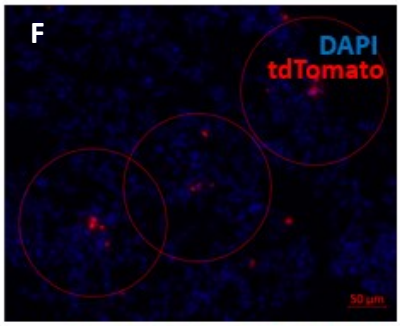

A
DAPI
CD11c
Combined
tdTomato
CD24

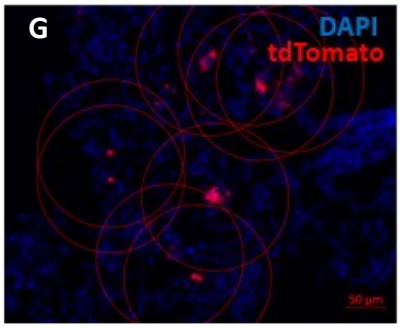

B
Comparison Between CD11c+ and CD11c+ CD24+ Cells Surrounding a Metastasis
# of Cells
25
20
15
10
5
0
CD11c+
CD11c+ CD24+
Cell Types


C
Proportions of Metastases According to # of Cells Inside
14.50%
10.14%
75.36%
Cell # in a Metastasis
1
2-10
>10
Total=138


D
Correlation Between # of Cells in a Metastasis and CD11c+ Cells Surrounding
# of CD11c+ Cells Surrounding
Y = 0.02207*X + 5.754
# of Cells in a Metastasis


E
Correlation Between # of Cells in a Metastasis and CD11c+ CD24+ Cells Surrounding
# of CD11c+ CD24+ Cells Surrounding
Y = -0.01465*X + 4.094
# of Cells in a Metastasis


F
DAPI
tdTomato
50 µm


G
DAPI
tdTomato
50 µm

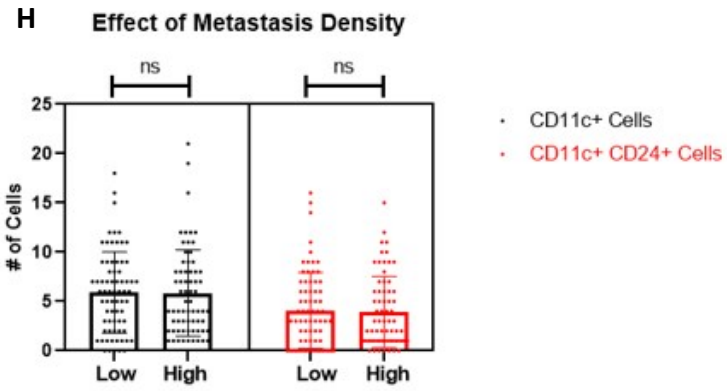

H
Effect of Metastasis Density
ns
ns
25
20
15
10
5
0
# of Cells
Low
High
Low
High
CD11c+ Cells
CD11c+ CD24+ Cells

Figure 5 Quantification of CD11c+ CD24+ Dendritic Cells Surrounding Metastases

(A) Determination of CD11c+ and CD11c+ CD24+ cells: CD11c+ cells are marked with yellow squares and CD11c+ CD24+ cells are marked with red squares. CD11c+ CD24+ cells are cells that are within 100 μm away from a metastasis, with nuclei stained with DAPI, membranes stained with CD11c but not tdTomato, and CD24 staining should be at least partially colocalized with CD11c+ membranes. Therefore, cells pointed by white arrows are not consider CD24+ although there are CD24 staining over cells, while cells pointed by yellow arrows are considered CD11c+ CD24+ because they have membranes stained with CD11c and at least partially stained with CD24.

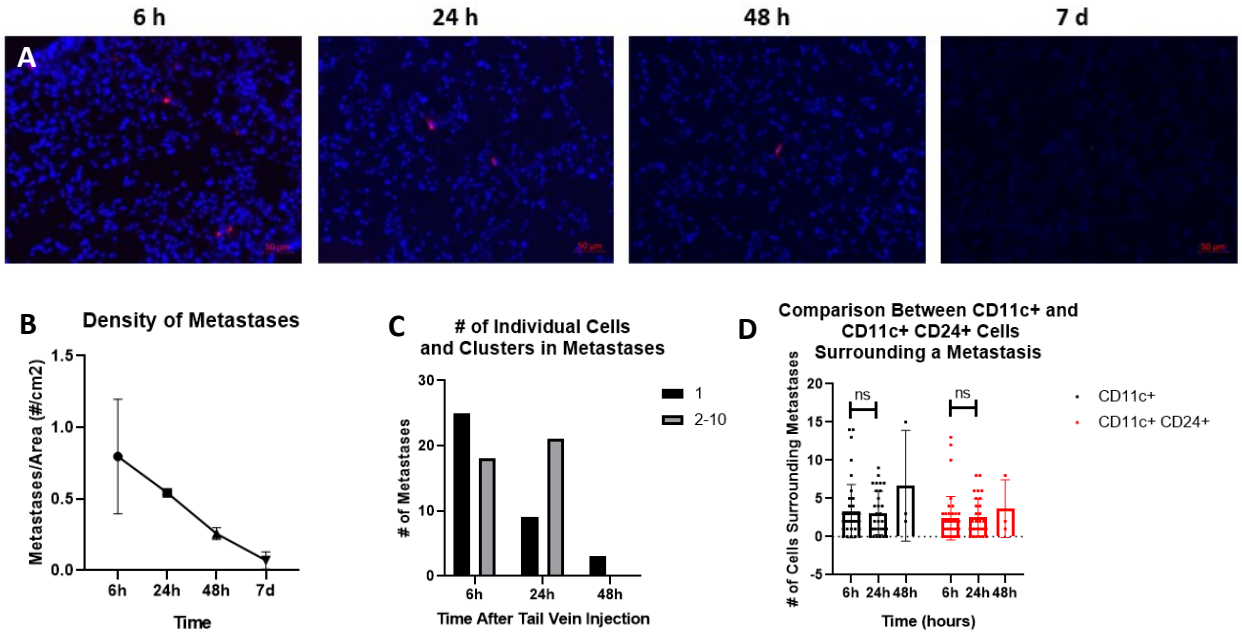
(B) Comparison Between CD11c+ and CD11c+ CD24+ Cells Surrounding a Metastasis (CD11c+ CD24+ cells are included in CD11c+ cells. Data are presented as mean ± SD).

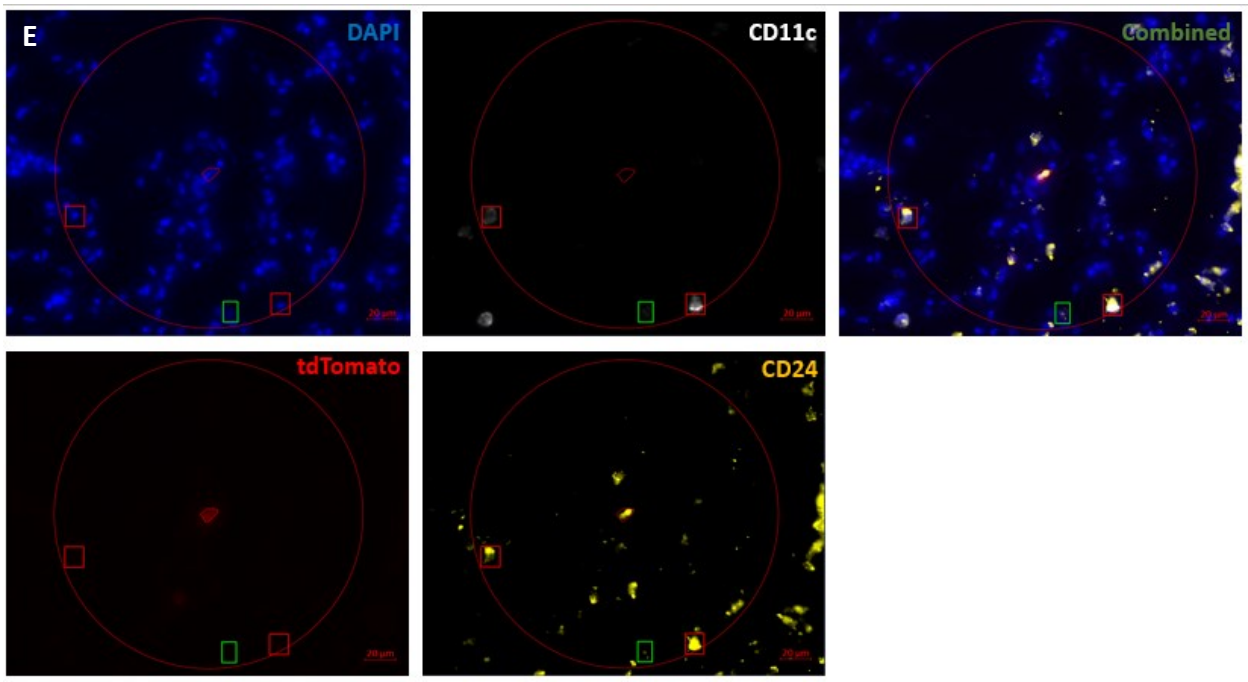
(C) Proportions of metastases according to numbers of cells inside.

(D-E) Correlation between numbers of cells in a metastasis and CD11c+ cells (D) or CD11c+ CD24+ cells surrounding it.

(F-G) Examples of metastases distributed in low density (F), where distances between metastases are larger than 100 μm, or in high density (G) where distances between multiple metastases are smaller than 100 μm.

(H) Comparison of CD11c+ or CD11c+ CD24+ cells surrounding metastases distributed in low and high density. (Data are presented as mean ± SD, Welch's t test, $p = 0.9290$ for CD11c+ cells, $p = 0.8028$ for CD11c+ CD24+ cells).


6 h
24 h
48 h
7 d
A
B
Density of Metastases
Metastases/Area (#/cm2)
1.5
1.0
0.5
0.0
6h
24h
48h
7d
Time
C
# of Individual Cells
and Clusters in Metastases
# of Metastases
30
20
10
0
1
2-10
6h
24h
48h
Time After Tail Vein Injection
D
Comparison Between CD11c+ and
CD11c+ CD24+ Cells
Surrounding a Metastasis
# of Cells Surrounding Metastases
20
15
10
5
0
-5
ns
ns
6h 24h 48h 6h 24h 48h
Time (hours)
CD11c+
CD11c+ CD24+


E
DAPI
CD11c
Combined
tdTomato
CD24

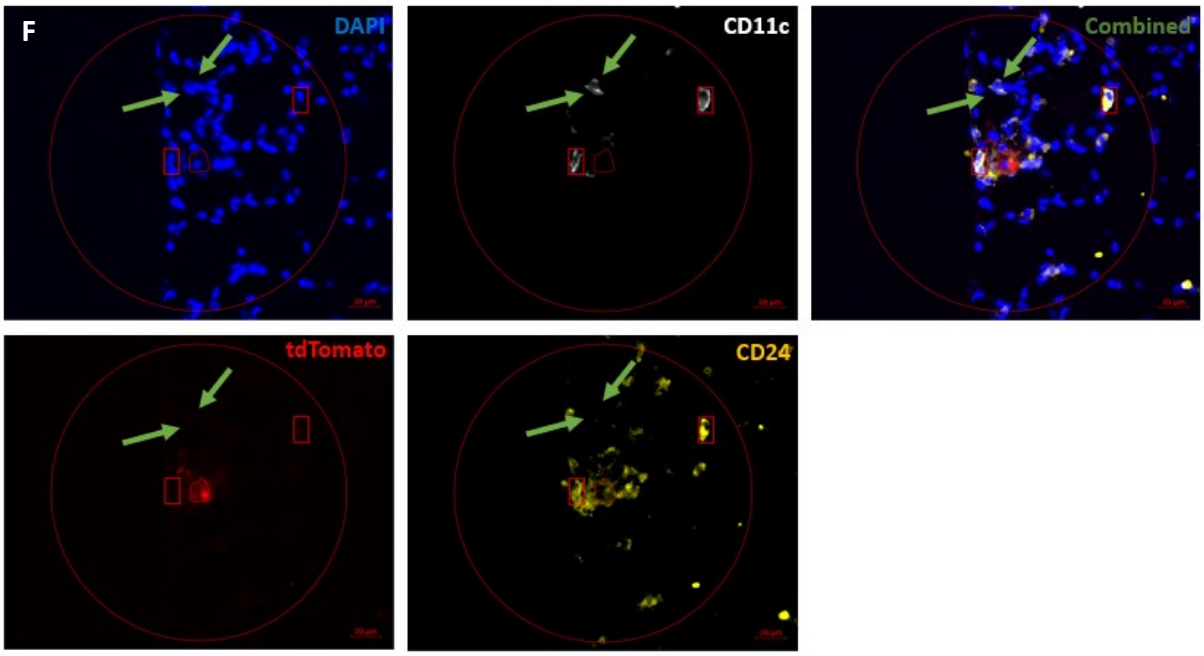

F
DAPI
CD11c
Combined
tdTomato
CD24

Figure 6 Dynamics of Metastases and CD11c+ or CD11c+ CD24+ Cells between 6 h and 7 days

(A) Examples of lung sections 6 h, 24 h, 48 h, and 7 d after tail vein injection.

(B) Densities of metastases on sections from lungs dissected 6 h, 24 h, 48 h, and 7 d after tail vein injection.

(C) Numbers of individual cells and clusters on sections from lungs dissected at different time points stained with CD11c and CD24.

(D) Numbers of CD11c+ and CD24+ cells surrounding a metastasis in lungs dissected 6 h, 24h, and 7 d after tail vein injection (Data are presented as mean ± SD, Welch's t test, $p = 0.7385$ for CD11c+ cells, $p = 0.8964$ for CD11c+ CD24+ cells).

(E) An example from a 6 h slide (a CD11c+ cell is surrounded by a green square, and two CD24+ CD11c+ cells are surrounded by red squares).

(F) An example from a 24 h slide (two CD11c+ cells are highlighted by green arrows, and two CD24+ CD11c+ cells are surrounded by red squares).

Discussion

In these experiments, we investigated the involvement of DCs in early stages of breast cancer metastasis. We found particles stained with CD11c colocalized with tdTomato-labeled metastases in lungs. Those particles appear to be located inside metastatic cells rather than on cellular membranes. We characterized those particles by double staining of CD11c and CD24, in order to determine the sources of those particles, or by staining of CD11c and Arf6, an ectosome marker, in order to determine the types of those particles. Then, the involvement of DCs in immune response against metastases was investigated by counting CD11c+CD24+ cells surrounding tdTomato-expressing clusters. The comparison between numbers of CD11c+ and CD11c+CD24+ cells surrounding metastases showed necessity of using another marker besides CD11c to prevent influence of other CD11c+ cells in research relevant to DCs. We tried to correlate the number of DCs surrounding a metastasis with the size or density of metastases in the region. Finally, we tried to see the dynamics of DCs surrounding colonizing metastases by quantifying CD11c+ and CD11c+CD24+ cells around metastases in lungs dissected 6h, 24h, 48h, or 7 days after tail vein injection.

CD11c+ Particles

In the experiment, particles stained by CD11c were found inside metastases in lungs after tail vein injection. Although CD11c is a classical marker for dendritic cells and CD11c+ extracellular vesicles derived from CD8α+ DCs have been found in mouse lungs [139], whether the CD11c+ found in this experiment requires verification, because in mouse lungs, tissue-resident macrophages can also express CD11c and release ectosomes during inflammation or cancer besides DCs [140], [141]. Furthermore, macrophages have been found to transfer cytoplasmic content to melanoma cells through direct contact between cellular membranes [125]. Therefore, we stained lung sections with the antibody against CD24, a marker to distinguish DCs and macrophages, besides the CD11c antibody to investigate the sources of CD11c+ particles.

Available data suggest that most CD11c+ particles did not co-stain with the CD24 antibody, so they may not have been released by dendritic cells. For example, CD24+ EVs can be secreted by normal tubule cells and podocytes in the kidneys, B-cells, ovarian carcinoma cells and T-cell lymphoma cells [142]-[145]. Therefore, this molecule can be considered releasable via EVs. The possibility that the CD11c+ particles are derived from DCs is thus reduced. In future, macrophage markers such as F4/80 may be co-stained with CD11c to investigate whether those particles are released by CD11c+ macrophages rather than DCs.

We also tried to characterize the types of CD11c+ particles besides their sources. Since their sizes ranged between 0.5 μm and 6 μm in diameter, those particles were not considered to be exosomes, which should be smaller than 0.15 μm, but a number of them meet the size criteria of ectosomes (0.1 – 1 μm) or apoptotic bodies (0.05 – 5 μm). In the experiment, we tried to verify whether at least some of the CD11c+ particles were ectosomes by staining them with the antibody against Arf6 besides CD11c.

We cannot draw a definitive conclusion about whether CD11c+ particles are ectosomes through staining of Arf6. The particles found on the section stained with anti-CD11c and anti-Arf6 antibodies were quite few. Many metastases were found expressing Arf6 at a high level, especially those found with CD11c+ particles, making determination of Arf6+ particles more difficult. Arf6 is found to be significantly correlated with metastases of multiple cancers including breast cancer [146], so it may not be a good marker for ectosomes in this research because of high background caused by cellular expression of this protein. Considering that many markers to label ectosomes are either cytosolic proteins which can also be expressed in recipient cells or are membranous proteins which can also be found in other EVs such as apoptotic bodies, the issue of high background may be inevitable in ectosome research that relies on tissue immunofluorescence staining. Therefore, a good strategy is to digest dissected lungs from injected mice into single cells, isolate tdTomato+ metastatic cells through FACS, digest isolated malignant cells and isolate CD11c+ particles through FACS again. Then those isolated particles can be

characterized by staining of relevant markers and flow cytometry. RNA-seq can also be performed to characterize potential microRNA contents inside those particles.

Since many CD11c+ particles also meet size criteria for apoptotic bodies, markers for apoptotic bodies can be stained to characterize the CD11c+ particles. The TUNEL assay to stain terminals of DNA fragments has been reported to detect apoptotic bodies on a tissue section [147]. Therefore, in the future, this assay may also be used to further investigate such particles.

Furthermore, detergents such as Triton X-100 can permeabilize cellular membranes so that antibodies can infiltrate into cells to stain intracellular proteins [135], but they may also damage cellular membranes, leading to formation of artifacts which were not seen on slides stained without permeabilization. CD11c+ particles may be such artifacts caused by permeabilization. In order to clarify whether the CD11c+ particles are artifacts resulting from the Triton X-100 treatment, sections of primary tumors stained with the anti-CD11c+ antibody or a control antibody with or without permeabilization can be compared. If CD11c+ particles can be found in complete staining without Triton-X but not in membranous staining without Triton-X, CD11c+ particles are more likely to be artifacts.

CD11c+ Staining on Metastases and Permeabilization in Immunofluorescence Staining

There is no report that cancer cells express CD11c. However, many metastases in the research were found to be stained by the anti-CD11c antibody, either in the form of CD11c+ particles or dispersed staining. However, staining without permeabilization via Triton X-100 was found to reduce such staining by CD11c+, while the staining of CD24 was not reduced. It seems that the CD11c staining was inside metastatic cells while the CD24 staining was on cellular membranes. It is noteworthy that in contrast to CD11c, CD24 is found to be expressed in multiple malignancies including breast cancer [148].

The use of Triton X-100 for permeabilization can lead to loss of cell surface proteins [137]. Since both CD11c and CD24 are surface markers expressed on cellular membranes, membranous staining without Triton X-100 was applied in later research relevant to CD11c+ and CD11c+ CD24+ cells.

It is possible that the CD11c+ staining in metastases are artifacts caused by Triton-X permeabilization, similar to CD11c+ particles. Comparison of primary tumor sections stained with the CD11c antibody and a control antibody can help verify whether the CD11c+ staining in metastases is a true signal.

Co-staining of CD11c and CD24 Rather Than CD11c for Labeling of Dendritic Cells

The double staining of lung sections with CD11c and CD24 demonstrates that not all CD11c+ cells express CD24. It supports previous research done through flow cytometry relevant to the usage of CD11c as a general marker of DCs in the form of immunostaining: not only DCs express CD11c in mouse lungs [95]. Therefore, results from previous research done with CD11c as the only label for DCs should be taken with caution. In the future, macrophage markers such as F4/80 can be labeled along with CD11c and CD24 to see whether the CD11c-expressing cells in lungs are composed of DCs and macrophages. Considering the finding that mouse DCs downregulate expression of CD11c upon activation [149], the use of additional markers with CD11c to stain DC markers should be performed.

CD11c+ and CD11c+ CD24+ Cells Surrounding Metastases

According to results of membranous staining of lungs dissected 2 h after tail vein injection, the number of CD11c+ or CD11c+ CD24+ cells surrounding metastases was not found to correlate with metastasis size or density. Therefore, whether CD11c+ or CD11c+ CD24+ cells are recruited to metastases requires verification. Densities of stained cells surrounding metastases, not surrounding metastases and in normal lungs may be compared to see whether dendritic cells, lung-resident or just arriving from circulation, accumulate to metastases and lungs with metastases in general. Since DCs can be divided into multiple subtypes, certain subtypes such as cDC1s are found to be more relevant to immunity

against cancer [48], and cancer cells can induce phenotypical changes of DCs [85], research focusing on roles of certain subtypes can be implemented. Lung DCs can be divided into two subtypes: CD103+ CD11b- and CD103- CD11b+, and during inflammation, monocytes can be differentiated to DCs, expressing CD11b and Ly6C [150]. The number of fluorescence channels on an imaging device limits the number of immunofluorescent markers that can be assessed at one time.

Dynamics of CD11c+ or CD11c+ CD24+ Cells Surrounding Metastases

The current results from slides of lungs dissected 6-h and 24-h do not show change in numbers of CD11c+ or CD11c+ CD24+ cells surrounding metastases. Besides the aforementioned issues of DC recruitment by metastases and involvement of different DC subtypes, the time points used in the research may be too late for such research concentrated on early colonization of metastases. Because of difference in numbers of cancer clusters injected into the 2-h mouse and other mice (one million versus 250 thousand), numbers of stained cells surrounding metastases cannot be directly compared between data from the 2-h lungs and lungs dissected at other time points. However, comparison among slides of lungs dissected 6 h, 24 h, 48 h, and 7 d after tail vein injection has shown a decrease of metastasis density. One of possible explanations is that the immune system, especially cytotoxic cells recruited by dendritic cells, may have engaged in immune responses against metastases at those time points. Therefore, in the future, lungs can be dissected sooner after cluster injection, at time points such as 30 min, 1 h, 2 h, 4 h, and 6 h, so that earlier stages of infiltration of metastases into lungs can be analyzed.

Utilization of Tail Vein Injection in a Metastasis Assay for Immune Research

Animal models of metastasis created by tail vein injection of malignant cells have been applied in multiple experiments and many theories of metastasis are established based on results gathered from such research. However, such assay cannot model the influence of primary tumors onto metastasis, including concomitant immunity against cancer of the whole body and influence of secretions of tumors onto metastatic organs before colonization [58][116]. Therefore, orthotopic transplant of tdTomato+

tumors can be done besides the assay with tail vein injection. The phenotypes of DCs at the very beginning of colonization can be compared between the two assays to see whether existence of a primary tumor has influence on DCs in a metastatic organ.

Limitations

We admit that the sample sizes of experiments involved in the thesis are small. Only 19 slides were analyzed in the research, with 5 sections from lungs of the mouse dissected 2 h after tail vein injection, 4 sections from the 6-h, 24-h, and 48-h mice respectively, and 2 sections from the 7-d mouse. Sections from the same mouse also showed great diversity in size and metastasis number and size. Furthermore, three slides involving CD11c+ particles showed great variety in percentages of metastases with particles and numbers of overlapping particles. Therefore, more mice should be dissected for each condition and more sections for each mouse should be analyzed so that the conclusions drawn from the research can be sufficiently supported.

Furthermore, the problem of non-specific staining occurred to immunofluorescence staining in the experiment, especially in the case of the anti-CD24 antibody, as shown in Figure 5A. Although staining was later improved by reducing the amount to antibodies used and washing slides with one more turn, as shown in Figures 6E and 6F, such changes in immunofluorescence procedures inevitably led to reduced fluorescence intensity and the threshold used to distinguish true signals from autofluorescence.

Methods

Mouse Lines and Breeding

All procedures performed relevant to mice were in accordance with protocols approved by the Johns Hopkins Medical Institute Animal Care and Use Committee (ACUC). All mice were female and were backcrossed and maintained on the FVB/n background. In the experiment, 6 wild type FVB mice, 1 MMTV::PyMT; (R26)pCA::mT/mG mouse and 1 MMTV::PyMT; (R26)pCA::mT/mG; K14::actin-GFP were used. The MMTV::PyMT; (R26)pCA::mT/mG mouse is a crossbreed of mouse lines of MMTV-PyMT [134], R26::Cre-ER [151], and mT/mG [152]. MMTV::PyMT; (R26)pCA::mT/mG; K14::actin-GFP is a crossbreed of mouse lines of the former three and K14-GFP-actin [153]. MMTV-PyMT34 and mT/mG mice were obtained from the Jackson Laboratories. The R26::Cre-ER mouse line was a gift from J. Nathans (Johns Hopkins University). K14-actin-GFP mice were a gift from Elaine Fuchs (Rockefeller University, New York, NY).

Isolation of Primary Mammary Gland Organoids

The tumor clusters used in the experiment were derived from trypsinization of primary tumor organoids, which were isolated from mouse primary mammary tumors according to well-established protocols of the lab [154]. Tumors were dissected from 10-12-week-old MMTV-PyMT mice carrying a ROSAmT/mG reporter or both the ROSAmT/mG reporter and a K14-GFP reporter. Then, tumors were mechanically minced with a scalpel and enzymatically digested in a shaking incubator (Thermo Scientific MaxQ 4450) at 110-150 rpm for 1 h at 37°C in collagenase solution, which was prepared by adding 2mg/mL collagenase (C2139; Sigma-Aldrich), 2 mg/mL trypsin (27250–018; Gibco Life Technologies), 5% (vol/vol) FBS (F0926; Sigma-Aldrich), 5 μg/mL insulin (I9278; Sigma-Aldrich), and 50 μg/mL gentamicin (15750; Gibco Life Technologies) to DMEM/F12 (10565–018; Gibco Life Technologies). After removal of fat and debris through centrifugation at 1500 rpm for 10 min, the suspension was treated with 2 U/μL DNase solution in PBS (without Ca2+ or Mg2+, 10010-023; Life Technologies) for dissociation of small clusters

into single cells. Large chunks of tissue were removed via natural precipitation. Organoids were isolated from single cells by 4 cycles of differential centrifugation: in each cycle, suspension was centrifuged at 1500 rpm for 3 s.

Tail Vein Injection

After washing with PBS, organoids were trypsinized into small cell clusters by incubating with TrypLE (12604013; Thermo Fisher) for 10 min at 37°C. Remaining organoids were filtered by pipetting suspension into 5 mL round bottom tubes (352235; Falcon) through their cell-strainer caps. The remaining clusters were counted and resuspended at a concentration of 10^6 or 4×10^6 cells/mL. They were transferred to 1.5 mL or 2 mL sterile Eppendorf tubes for use in tail vein injection.

Tumor clusters collected were injected into wildtype FVB mice intravenously. After putting a mouse into a restrainer, its tail was dilated by a heat lamp for 5 min. Then, for each mouse, 250 µL tumor cluster suspension was injected into its tail vein with a U-100 insulin syringe (329461; Becton Dickinson).

Collection and Fixation of Mouse Lungs

Lungs of injected mice were collected 2 h, 6 h, 24 h, 48 h, or a week after tail vein injection. After exposing the heart and the lungs of mice by cutting its rib cage, we perfused the mouse at least 10 mL 1% PFA (P6148; Sigma-Aldrich) solution in PBS through the right ventricle of the heart. Then at least 10mL PBS was injected at the same site to clear PFA solution. Then lungs were dissected with the heart, put in 1% PFA solution for fixation and incubated at 4°C overnight. After that, lungs were transferred to 25% sucrose (S7903; Sigma) solution in PBS and incubated at 4°C overnight. They were then embedded in Tissue-Tek OCT compound (4583; Sakura). The tissue samples embedded in OCT were sectioned into sections with thickness of 10mm at -30°C in a cryostat (Thermo Scientific Cryostar NX50).

Immunofluorescence Staining

In the experiment, two types of immunofluorescence techniques were used, to stain membranous or both intracellular and membranous proteins. In both techniques, tissue sections were covered by DPBS (with Ca^{2+} and Mg^{2+}, D8662; Sigma) for 1 h at room temperature to remove OCT. Then while in membranous staining sections were directly blocked with block buffer for 2 h at room temperature, in complete staining the section was permeabilized with 0.5% Triton X-100 (X100; Sigma) solution in PBS before blocking. Then sections were incubated with conjugated antibodies and primary antibodies diluted in dilution buffer overnight at 4°C. After being rinsed with PBS for 10 min 2-3 times, sections were incubated with DAPI and secondary antibodies diluted in dilution buffer for 4 h at room temperature. Then sections were rinsed with PBS for 10 min 3-5 times. Sections were then mounted and sealed with coverslips. The formulas for block and antibody dilution buffers are shown in Table 1.

Primary and conjugated antibodies used in the experiment were CD11c (dilution ratio 1:100, N418, conjugated with AF647, 117314; BioLegend), CD24 (dilution ratio 1:100-1:200, ab214231; Abcam), and ARF6 (dilution ratio 1:100, PA1-093; Invitrogen). Secondary antibodies were diluted with the ratio 1:200. DAPI was diluted with the ratio 1:1000.

Table 1 Composition of block and antibody dilution buffers in membranous and complete staining

	Block Buffer	Antibody Dilution Buffer
Membranous Staining	10% FBS, 1% BSA solution (30% in DPBS, A9576; Sigma-Aldrich) in PBS	1% FBS, 1% BSA solution in PBS
Complete Staining	10% FBS, 1% BSA solution, and 0.2% Triton X-100 in PBS	1% FBS, 1% BSA solution, and 0.2% Triton X-100 in PBS

Slide Scanning and Image and Statistical Analysis

Stained and sealed slides were scanned with a Zeiss Axio Scan.Z1 and an AxioCam MRM camera (Zeiss). Images were processed with Zeiss ZEN 3.1. Statistical analysis was performed using GraphPad Prism 8. Statistical tests used for each experiment and other parameters associated with the representation of the data are shown by figure legends.

References

[1] J. Ferlay *et al*, "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer,* vol. 136, *(5),* pp. E359-E386, 2015.

[2] C. E. DeSantis *et al*, "Breast cancer statistics, 2017, racial disparity in mortality by state," *CA: A Cancer Journal for Clinicians,* vol. 67, *(6),* pp. 439-448, 2017.

[3] T. Sørlie *et al*, "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications," *Proceedings of the National Academy of Sciences,* vol. 98, *(19),* pp. 10869-10874, 2001.

[4] N. Harbeck and M. Gnant, "Breast cancer," *The Lancet,* vol. 389, *(10074),* pp. 1134-1150, 2017. Available: https://doi.org/10.1016/S0140-6736(16)31891-8. DOI: 10.1016/S0140-6736(16)31891-8.

[5] X. Dai *et al*, "Breast cancer intrinsic subtype classification, clinical use and future trends," *American Journal of Cancer Research,* vol. 5, *(10),* pp. 2929, 2015.

[6] M. C. Cheang *et al*, "Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer," *JNCI: Journal of the National Cancer Institute,* vol. 101, *(10),* pp. 736-750, 2009.

[7] S. Turcotte and S. A. Rosenberg, "Immunotherapy for metastatic solid cancers," *Adv. Surg.,* vol. 45, pp. 341-360, 2011. Available: https://pubmed.ncbi.nlm.nih.gov/21954698 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3578602/. DOI: 10.1016/j.yasu.2011.04.003.

[8] R. L. Siegel, K. D. Miller and A. Jemal, "Cancer statistics, 2020," *CA: A Cancer Journal for Clinicians,* vol. 70, *(1),* pp. 7-30, 2020.

[9] A. G. Clark and D. M. Vignjevic, "Modes of cancer cell invasion and the role of the microenvironment," *Curr. Opin. Cell Biol.,* vol. 36, pp. 13-22, 2015.

[10] S. P. Chiang, R. M. Cabrera and J. E. Segall, "Tumor cell intravasation," *American Journal of Physiology-Cell Physiology,* vol. 311, *(1),* pp. C1-C14, 2016.

[11] K. Pantel and M. R. Speicher, "The biology of circulating tumor cells," *Oncogene,* vol. 35, *(10),* pp. 1216-1224, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/26050619. DOI: 10.1038/onc.2015.192.

[12] N. Reymond, B. B. d'Água and A. J. Ridley, "Crossing the endothelial barrier during metastasis," *Nature Reviews.Cancer,* vol. 13, *(12),* pp. 858-870, 2013. Available: https://pubmed.ncbi.nlm.nih.gov/24263189. DOI: 10.1038/nrc3628.

[13] J. Massagué and A. C. Obenauf, "Metastatic colonization by circulating tumour cells," *Nature,* vol. 529, *(7586),* pp. 298-306, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/26791720 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5029466/. DOI: 10.1038/nature17038.

[14] J. J. F. Sleeboom *et al*, "Metastasis in context: modeling the tumor microenvironment with cancer-on-a-chip approaches," *Dis Models Mech,* vol. 11, *(3),* pp. dmm033100, 2018. Available: http://dmm.biologists.org/content/11/3/dmm033100.abstract. DOI: 10.1242/dmm.033100.

[15] S. A. Narod and V. Sopik, "Is invasion a necessary step for metastases in breast cancer?" *Breast Cancer Res. Treat.,* vol. 169, *(1),* pp. 9-23, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29353366 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5882625/. DOI: 10.1007/s10549-017-4644-3.

[16] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell,* vol. 144, *(5),* pp. 646-674, 2011. Available: https://pubmed.ncbi.nlm.nih.gov/21376230. DOI: 10.1016/j.cell.2011.02.013.

[17] K. J. Cheung *et al*, "Collective invasion in breast cancer requires a conserved basal epithelial program," *Cell,* vol. 155, *(7),* pp. 1639-1651, 2013. Available: https://pubmed.ncbi.nlm.nih.gov/24332913 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3941206/. DOI: 10.1016/j.cell.2013.11.029.

[18] G. E. Moore, A. A. Sandberg and A. L. Watne, "The comparative size and structure of tumor cells and clumps in the blood, bone marrow, and tumor imprints," *Cancer,* vol. 13, *(1),* pp. 111-117, 1960. Available: https://doi.org/10.1002/1097-0142(196001/02)13:13.0.CO;2-Y. DOI: 10.1002/1097-0142(196001/02)13:13.0.CO;2-Y.

[19] K. J. Cheung *et al*, "Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters," *Proc. Natl. Acad. Sci. U. S. A.,* vol. 113, *(7),* pp. E854-E863, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/26831077 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4763783/. DOI: 10.1073/pnas.1508541113.

[20] M. Aubele *et al*, "Intratumoral Heterogeneity in Breast Carcinoma Revealed by Laser-Microdissection and Comparative Genomic Hybridization," *Cancer Genetics and Cytogenetics,* vol. 110, *(2),* pp. 94-102, 1999. Available: http://www.sciencedirect.com/science/article/pii/S0165460898002052. DOI: https://doi.org/10.1016/S0165-4608(98)00205-2.

[21] A. Pribluda, de la Cruz, Cecile C. and E. L. Jackson, "Intratumoral Heterogeneity: From Diversity Comes Resistance," *Clin. Cancer Res.,* vol. 21, *(13),* pp. 2916, 2015. Available: http://clincancerres.aacrjournals.org/content/21/13/2916.abstract. DOI: 10.1158/1078-0432.CCR-14-1213.

[22] P. B. Gupta *et al*, "Phenotypic Plasticity: Driver of Cancer Initiation, Progression, and Therapy Resistance," *Cell Stem Cell,* vol. 24, *(1),* pp. 65-78, 2019. Available: https://pubmed.ncbi.nlm.nih.gov/30554963. DOI: 10.1016/j.stem.2018.11.011.

[23] M. A. Nieto *et al*, "EMT: 2016," *Cell,* vol. 166, *(1),* pp. 21-45, 2016.

[24] C. L. Chaffer *et al*, "EMT, cell plasticity and metastasis," *Cancer Metastasis Rev.,* vol. 35, *(4),* pp. 645-654, 2016.

[25] R. Bill and G. Christofori, "The relevance of EMT in breast cancer metastasis: Correlation or causality?" *FEBS Lett.,* vol. 589, *(14),* pp. 1577-1587, 2015.

[26] K. R. Fischer *et al*, "Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance," *Nature,* vol. 527, *(7579),* pp. 472-476, 2015. Available: https://pubmed.ncbi.nlm.nih.gov/26560033 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4662610/. DOI: 10.1038/nature15748.

[27] V. Padmanaban *et al*, "E-cadherin is required for metastasis in multiple models of breast cancer," *Nature,* vol. 573, *(7774),* pp. 439-444, 2019. Available: https://doi.org/10.1038/s41586-019-1526-3. DOI: 10.1038/s41586-019-1526-3.

[28] N. M. Aiello *et al*, "EMT subtype influences epithelial plasticity and mode of cell migration," *Developmental Cell,* vol. 45, *(6),* pp. 681-695. e4, 2018.

[29] Y. Gao *et al*, "Metastasis organotropism: redefining the congenial soil," *Developmental Cell,* vol. 49, *(3),* pp. 375-391, 2019.

[30] C. W. Wong *et al*, "Apoptosis: an early event in metastatic inefficiency," *Cancer Res.,* vol. 61, *(1),* pp. 333-338, 2001. Available: https://pubmed.ncbi.nlm.nih.gov/11196183.

[31] M. S. Sosa, P. Bragado and J. Aguirre-Ghiso, "Mechanisms of disseminated cancer cell dormancy: an awakening field," *Nature Reviews.Cancer,* vol. 14, *(9),* pp. 611-622, 2014. Available: https://pubmed.ncbi.nlm.nih.gov/25118602 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4230700/. DOI: 10.1038/nrc3793.

[32] Y. Liu and X. Cao, "Characteristics and Significance of the Pre-metastatic Niche," *Cancer Cell,* vol. 30, *(5),* pp. 668-681, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/27846389. DOI: 10.1016/j.ccell.2016.09.011.

[33] D. F. Quail and J. A. Joyce, "Microenvironmental regulation of tumor progression and metastasis," *Nat. Med.,* vol. 19, *(11),* pp. 1423, 2013.

[34] H. Gonzalez, C. Hagerling and Z. Werb, "Roles of the immune system in cancer: from tumor initiation to metastatic progression," *Genes Dev.,* vol. 32, *(19-20),* pp. 1267-1284, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/30275043 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6169832/. DOI: 10.1101/gad.314617.118.

[35] A. Ponomarev and I. Shubina, "Insights into mechanisms of tumor and immune system interaction: association with wound healing," *Frontiers in Oncology,* vol. 9, pp. 1115, 2019.

[36] S. D. Vallabhapurapu *et al*, "Variation in human cancer cell external phosphatidylserine is regulated by flippase activity and intracellular calcium," *Oncotarget,* vol. 6, *(33),* pp. 34375-34388, 2015. Available: https://pubmed.ncbi.nlm.nih.gov/26462157 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4741459/. DOI: 10.18632/oncotarget.6045.

[37] M. Zamanian *et al*, "Calreticulin and cancer," *Pathology & Oncology Research,* vol. 19, *(2),* pp. 149-154, 2013.

[38] J. Wu and L. L. Lanier, "Natural killer cells and cancer," *Adv. Cancer Res.,* vol. 90, pp. 127-156, 2003. Available: https://pubmed.ncbi.nlm.nih.gov/14710949. DOI: 10.1016/s0065-230x(03)90004-2.

[39] C. Boudesco *et al*, "Hsp70: A Cancer Target Inside and Outside the Cell," *Methods Mol. Biol.,* vol. 1709, pp. 371-396, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29177673. DOI: 10.1007/978-1-4939-7477-1_27.

[40] Y. Chen *et al*, "The B7 Family Member B7-H6: a New Bane of Tumor," *Pathology Oncology Research : POR,* vol. 24, *(4),* pp. 717-721, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29086181. DOI: 10.1007/s12253-017-0357-5.

[41] P. Dhar and J. D. Wu, "NKG2D and its ligands in cancer," *Curr. Opin. Immunol.,* vol. 51, pp. 55-61, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29525346 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6145810/. DOI: 10.1016/j.coi.2018.02.004.

[42] T. Jiang *et al*, "Tumor neoantigens: from basic research to clinical applications," *Journal of Hematology & Oncology,* vol. 12, *(1),* pp. 93, 2019. Available: https://doi.org/10.1186/s13045-019-0787-5. DOI: 10.1186/s13045-019-0787-5.

[43] H. T. Wepsic, "Overview of oncofetal antigens in cancer," *Ann. Clin. Lab. Sci.,* vol. 13, *(4),* pp. 261-266, 1983. Available: https://pubmed.ncbi.nlm.nih.gov/6194734.

[44] M. F. Gjerstorff, M. H. Andersen and H. J. Ditzel, "Oncogenic cancer/testis antigens: prime candidates for immunotherapy," *Oncotarget,* vol. 6, *(18),* pp. 15772-15787, 2015. Available: https://pubmed.ncbi.nlm.nih.gov/26158218 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4599236/. DOI: 10.18632/oncotarget.4694.

[45] S. Spranger and T. F. Gajewski, "Mechanisms of Tumor Cell–Intrinsic Immune Evasion," *Annu. Rev. Cancer Biol.,* vol. 2, *(1),* pp. 213-228, 2018. Available: https://doi.org/10.1146/annurev-cancerbio-030617-050606. DOI: 10.1146/annurev-cancerbio-030617-050606.

[46] G. L. Beatty and W. L. Gladney, "Immune Escape Mechanisms as a Guide for Cancer Immunotherapy," *Clin. Cancer Res.,* vol. 21, *(4),* pp. 687, 2015. Available: http://clincancerres.aacrjournals.org/content/21/4/687.abstract. DOI: 10.1158/1078-0432.CCR-14-1860.

[47] G. P. Dunn *et al*, "Cancer immunoediting: from immunosurveillance to tumor escape," *Nat. Immunol.,* vol. 3, *(11),* pp. 991-998, 2002.

[48] J. Böttcher P. *et al*, "NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control," *Cell,* vol. 172, *(5),* pp. 1022-1037.e14, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29429633 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5847168/. DOI: 10.1016/j.cell.2018.01.004.

[49] D. R. Leach, M. F. Krummel and J. P. Allison, "Enhancement of Antitumor Immunity by CTLA-4 Blockade," *Science,* vol. 271, *(5256),* pp. 1734, 1996. Available: http://science.sciencemag.org/content/271/5256/1734.abstract. DOI: 10.1126/science.271.5256.1734.

[50] Y. Iwai *et al*, "Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade," *Proceedings of the National Academy of Sciences,* vol. 99, *(19),* pp. 12293-12297, 2002.

[51] F. Fallarino *et al*, "Modulation of tryptophan catabolism by regulatory T cells," *Nat. Immunol.,* vol. 4, *(12),* pp. 1206-1212, 2003. Available: https://pubmed.ncbi.nlm.nih.gov/14578884. DOI: 10.1038/ni1003.

[52] C. Smith *et al*, "IDO is a nodal pathogenic driver of lung cancer and metastasis development," *Cancer Discovery,* vol. 2, *(8),* pp. 722-735, 2012. Available: https://pubmed.ncbi.nlm.nih.gov/22822050 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3677576/. DOI: 10.1158/2159-8290.CD-12-0014.

[53] G. C. Prendergast *et al*, "Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer," *Cancer Immunology, Immunotherapy : CII,* vol. 63, *(7),* pp. 721-735, 2014. Available: https://pubmed.ncbi.nlm.nih.gov/24711084 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4384696/. DOI: 10.1007/s00262-014-1549-4.

[54] H. Roca *et al*, "CCL2 and interleukin-6 promote survival of human CD11b peripheral blood mononuclear cells and induce M2-type macrophage polarization," *J. Biol. Chem.,* vol. 284, *(49),* pp. 34342-34354, 2009.

[55] B. Qian and J. W. Pollard, "Macrophage diversity enhances tumor progression and metastasis," *Cell,* vol. 141, *(1),* pp. 39-51, 2010. Available: https://pubmed.ncbi.nlm.nih.gov/20371344 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4994190/. DOI: 10.1016/j.cell.2010.03.014.

[56] L. M. Janssen *et al*, "The immune system in cancer metastasis: friend or foe?" *Journal for Immunotherapy of Cancer,* vol. 5, *(1),* pp. 79, 2017.

[57] J. Eyles *et al*, "Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma," *J. Clin. Invest.,* vol. 120, *(6),* pp. 2030-2039, 2010. Available: https://pubmed.ncbi.nlm.nih.gov/20501944 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2877955/. DOI: 10.1172/JCI42002.

[58] R. A. Ruggiero *et al*, "Antitumor concomitant immunity: a possible metastasis control mechanism," *Medicina,* vol. 49, *(3),* pp. 277-281, 1989. Available: https://pubmed.ncbi.nlm.nih.gov/2487422.

[59] T. R. Cox *et al*, "The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase," *Nature,* vol. 522, *(7554),* pp. 106-110, 2015. Available: https://doi.org/10.1038/nature14492. DOI: 10.1038/nature14492.

[60] Y. Wang *et al*, "MDSCs: Key Criminals of Tumor Pre-metastatic Niche Formation," *Frontiers in Immunology,* vol. 10, pp. 172, 2019. Available: https://pubmed.ncbi.nlm.nih.gov/30792719 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6374299/. DOI: 10.3389/fimmu.2019.00172.

[61] S. K. Wculek and I. Malanchi, "Neutrophils support lung colonization of metastasis-initiating breast cancer cells," *Nature,* vol. 528, *(7582),* pp. 413-417, 2015. Available: https://doi.org/10.1038/nature16140. DOI: 10.1038/nature16140.

[62] C. Théry and S. Amigorena, "The cell biology of antigen presentation in dendritic cells," *Curr. Opin. Immunol.,* vol. 13, *(1),* pp. 45-51, 2001. Available: https://pubmed.ncbi.nlm.nih.gov/11154916. DOI: 10.1016/s0952-7915(00)00180-1.

[63] A. Alloatti *et al*, "Dendritic cell maturation and cross-presentation: timing matters!" *Immunol. Rev.,* vol. 272, *(1),* pp. 97-108, 2016. Available: https://doi.org/10.1111/imr.12432. DOI: 10.1111/imr.12432.

[64] L. A. Harshyne *et al*, "A Role for Class A Scavenger Receptor in Dendritic Cell Nibbling from Live Cells," *J. Immunol.,* vol. 170, *(5),* pp. 2302, 2003. Available: http://www.jimmunol.org/content/170/5/2302.abstract. DOI: 10.4049/jimmunol.170.5.2302.

[65] W. S. Garrett *et al*, "Developmental control of endocytosis in dendritic cells by Cdc42," *Cell,* vol. 102, *(3),* pp. 325-334, 2000.

[66] C. Reis e Sousa, "Dendritic cells in a mature age," *Nature Reviews Immunology,* vol. 6, *(6),* pp. 476-483, 2006. Available: https://doi.org/10.1038/nri1845. DOI: 10.1038/nri1845.

[67] J. M. Austyn *et al*, "Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone." *J. Exp. Med.,* vol. 167, *(2),* pp. 646-651, 1988.

[68] P. Vargas *et al*, "Innate control of actin nucleation determines two distinct migration behaviours in dendritic cells," *Nat. Cell Biol.,* vol. 18, *(1),* pp. 43-53, 2016.

[69] J. M. Blander, "Regulation of the cell biology of antigen cross-presentation," *Annu. Rev. Immunol.,* vol. 36, pp. 717-753, 2018.

[70] C. Macri *et al*, "Dendritic cell subsets," *Semin. Cell Dev. Biol.,* vol. 84, pp. 11-21, 2018. Available: http://www.sciencedirect.com/science/article/pii/S1084952116304347. DOI: https://doi.org/10.1016/j.semcdb.2017.12.009.

[71] M. Collin and V. Bigley, "Human dendritic cell subsets: an update," *Immunology,* vol. 154, *(1),* pp. 3-20, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29313948 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5904714/. DOI: 10.1111/imm.12888.

[72] L. Zhou and T. F. Tedder, "CD14 blood monocytes can differentiate into functionally mature CD83 dendritic cells," *Proceedings of the National Academy of Sciences,* vol. 93, *(6),* pp. 2588-2592, 1996.

[73] T. Vu Manh *et al*, "Investigating Evolutionary Conservation of Dendritic Cell Subset Identity and Functions," *Frontiers in Immunology,* vol. 6, pp. 260, 2015. Available: https://pubmed.ncbi.nlm.nih.gov/26082777 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4451681/. DOI: 10.3389/fimmu.2015.00260.

[74] D. Dudziak *et al*, "Differential antigen processing by dendritic cell subsets in vivo," *Science (New York, N.Y.),* vol. 315, *(5808),* pp. 107-111, 2007. Available: https://pubmed.ncbi.nlm.nih.gov/17204652. DOI: 10.1126/science.1136080.

[75] F. P. Siegal *et al*, "The nature of the principal type 1 interferon-producing cells in human blood," *Science (New York, N.Y.),* vol. 284, *(5421),* pp. 1835-1837, 1999. Available: https://pubmed.ncbi.nlm.nih.gov/10364556. DOI: 10.1126/science.284.5421.1835.

[76] A. Gardner and B. Ruffell, "Dendritic Cells and Cancer Immunity," *Trends Immunol.,* vol. 37, *(12),* pp. 855-865, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/27793569 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5135568/. DOI: 10.1016/j.it.2016.09.006.

[77] D. Chen and I. Mellman, "Oncology Meets Immunology: The Cancer-Immunity Cycle," *Immunity,* vol. 39, *(1),* pp. 1-10, 2013. Available: http://www.sciencedirect.com/science/article/pii/S1074761313002963. DOI: https://doi.org/10.1016/j.immuni.2013.07.012.

[78] K. C. Barry *et al*, "A natural killer–dendritic cell axis defines checkpoint therapy–responsive tumor microenvironments," *Nat. Med.,* vol. 24, *(8),* pp. 1178-1191, 2018. Available: https://doi.org/10.1038/s41591-018-0085-8. DOI: 10.1038/s41591-018-0085-8.

[79] R. Noubade, S. Majri-Morrison and K. V. Tarbell, "Beyond cDC1: Emerging Roles of DC Crosstalk in Cancer Immunity," *Frontiers in Immunology,* vol. 10, pp. 1014, 2019. Available: https://pubmed.ncbi.nlm.nih.gov/31143179 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6521804/. DOI: 10.3389/fimmu.2019.01014.

[80] J. Tel *et al*, "Natural Human Plasmacytoid Dendritic Cells Induce Antigen-Specific T-Cell Responses in Melanoma Patients," *Cancer Res.,* vol. 73, *(3),* pp. 1063, 2013. Available: http://cancerres.aacrjournals.org/content/73/3/1063.abstract. DOI: 10.1158/0008-5472.CAN-12-2583.

[81] M. Gallotta *et al*, "Inhaled TLR9 Agonist Renders Lung Tumors Permissive to PD-1 Blockade by Promoting Optimal CD4(+) and CD8(+) T-cell Interplay," *Cancer Res.,* vol. 78, *(17),* pp. 4943-4956, 2018. Available: https://pubmed.ncbi.nlm.nih.gov/29945961. DOI: 10.1158/0008-5472.CAN-18-0729.

[82] M. Y. Gerner, K. A. Casey and M. F. Mescher, "Defective MHC class II presentation by dendritic cells limits CD4 T cell help for antitumor CD8 T cell responses," *Journal of Immunology (Baltimore, Md.: 1950),* vol. 181, *(1),* pp. 155-164, 2008. Available: https://pubmed.ncbi.nlm.nih.gov/18566380 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2587216/. DOI: 10.4049/jimmunol.181.1.155.

[83] S. Spranger, R. Bao and T. F. Gajewski, "Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity," *Nature,* vol. 523, *(7559),* pp. 231-235, 2015.

[84] A. Pinzon-Charry, T. Maxwell and J. A. López, "Dendritic cell dysfunction in cancer: A mechanism for immunosuppression," *Immunol. Cell Biol.,* vol. 83, *(5),* pp. 451-461, 2005. Available: https://doi.org/10.1111/j.1440-1711.2005.01371.x. DOI: 10.1111/j.1440-1711.2005.01371.x.

[85] U. K. Scarlett *et al*, "Ovarian cancer progression is controlled by phenotypic changes in dendritic cells," *J. Exp. Med.,* vol. 209, *(3),* pp. 495-506, 2012. Available: https://doi.org/10.1084/jem.20111413. DOI: 10.1084/jem.20111413.

[86] A. Sawant *et al*, "Depletion of plasmacytoid dendritic cells inhibits tumor growth and prevents bone metastasis of breast cancer cells," *The Journal of Immunology,* vol. 189, *(9),* pp. 4258-4265, 2012.

[87] J. A. Kenkel *et al*, "An Immunosuppressive Dendritic Cell Subset Accumulates at Secondary Sites and Promotes Metastasis in Pancreatic Cancer," *Cancer Res.,* vol. 77, *(15),* pp. 4158-4170, 2017. Available: https://pubmed.ncbi.nlm.nih.gov/28611041 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5550516/. DOI: 10.1158/0008-5472.CAN-16-2212.

[88] M. Merad *et al*, "The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting," *Annu. Rev. Immunol.,* vol. 31, pp. 563-604, 2013. Available: https://pubmed.ncbi.nlm.nih.gov/23516985 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3853342/. DOI: 10.1146/annurev-immunol-020711-074950.

[89] J. Wu *et al*, "Critical role of integrin CD11c in splenic dendritic cell capture of missing-self CD47 cells to induce adaptive immunity," *Proc. Natl. Acad. Sci. USA,* vol. 115, *(26),* pp. 6786-6791, 2018. Available: http://www.pnas.org/content/115/26/6786.abstract. DOI: 10.1073/pnas.1805542115.

[90] H. C. Probst *et al*, "Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells," *Clin. Exp. Immunol.,* vol. 141, *(3),* pp. 398-404, 2005. Available: https://pubmed.ncbi.nlm.nih.gov/16045728 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1809468/. DOI: 10.1111/j.1365-2249.2005.02868.x.

[91] J. P. Metlay *et al*, "The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies," *J. Exp. Med.,* vol. 171, *(5),* pp. 1753-1771, 1990. Available: https://pubmed.ncbi.nlm.nih.gov/2185332 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2187889/. DOI: 10.1084/jem.171.5.1753.

[92] J. Qualai *et al*, "Expression of CD11c Is Associated with Unconventional Activated T Cell Subsets with High Migratory Potential," *PloS One,* vol. 11, *(4),* pp. e0154253, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/27119555 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4847787/. DOI: 10.1371/journal.pone.0154253.

[93] T. Aranami, S. Miyake and T. Yamamura, "Differential Expression of CD11c by Peripheral Blood NK Cells Reflects Temporal Activity of Multiple Sclerosis," *J. Immunol.,* vol. 177, *(8),* pp. 5659, 2006. Available: http://www.jimmunol.org/content/177/8/5659.abstract. DOI: 10.4049/jimmunol.177.8.5659.

[94] S. L. Gibbings *et al*, "Three Unique Interstitial Macrophages in the Murine Lung at Steady State," *American Journal of Respiratory Cell and Molecular Biology,* vol. 57, *(1),* pp. 66-76, 2017. Available: https://pubmed.ncbi.nlm.nih.gov/28257233 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5516280/. DOI: 10.1165/rcmb.2016-0361OC.

[95] A. V. Misharin *et al*, "Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung," *American Journal of Respiratory Cell and Molecular Biology,* vol. 49, *(4),* pp. 503-510, 2013.

[96] A. Schlitzer *et al*, "IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses," *Immunity,* vol. 38, *(5),* pp. 970-983, 2013. Available:

https://pubmed.ncbi.nlm.nih.gov/23706669 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3666057/. DOI: 10.1016/j.immuni.2013.04.011.

[97] Y. R. Yu *et al*, "A Protocol for the Comprehensive Flow Cytometric Analysis of Immune Cells in Normal and Inflamed Murine Non-Lymphoid Tissues." *PloS One,* vol. 11, *(3),* pp. e0150606, 2016.

[98] G. van Niel, G. D'Angelo and G. Raposo, "Shedding light on the cell biology of extracellular vesicles," *Nature Reviews Molecular Cell Biology,* vol. 19, *(4),* pp. 213-228, 2018. Available: https://doi.org/10.1038/nrm.2017.125. DOI: 10.1038/nrm.2017.125.

[99] C. Harding, J. Heuser and P. Stahl, "Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding," *Eur. J. Cell Biol.,* vol. 35, *(2),* pp. 256-263, 1984. Available: https://pubmed.ncbi.nlm.nih.gov/6151502.

[100] C. Tricarico, J. Clancy and C. D'Souza-Schorey, "Biology and biogenesis of shed microvesicles," *Small GTPases,* vol. 8, *(4),* pp. 220-232, 2017. Available: https://pubmed.ncbi.nlm.nih.gov/27494381 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5680703/. DOI: 10.1080/21541248.2016.1215283.

[101] V. Muralidharan-Chari *et al*, "ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles," *Current Biology : CB,* vol. 19, *(22),* pp. 1875-1885, 2009. Available: https://pubmed.ncbi.nlm.nih.gov/19896381 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3150487/. DOI: 10.1016/j.cub.2009.09.059.

[102] J. Kowal *et al*, "Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes," *Proc. Natl. Acad. Sci. USA,* vol. 113, *(8),* pp. E968-E977, 2016. Available: http://www.pnas.org/content/113/8/E968.abstract. DOI: 10.1073/pnas.1521230113.

[103] S. Caruso and I. K. H. Poon, "Apoptotic Cell-Derived Extracellular Vesicles: More Than Just Debris," *Frontiers in Immunology,* vol. 9, pp. 1486, 2018. Available: https://www.frontiersin.org/article/10.3389/fimmu.2018.01486. DOI: 10.3389/fimmu.2018.01486.

[104] A. Becker *et al*, "Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis," *Cancer Cell,* vol. 30, *(6),* pp. 836-848, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/27960084 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5157696/. DOI: 10.1016/j.ccell.2016.10.009.

[105] F. Xie *et al*, "Extracellular Vesicles in Cancer Immune Microenvironment and Cancer Immunotherapy," *Advanced Science,* pp. 1901779, 2019.

[106] D. Di Vizio *et al*, "Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease," *Cancer Res.,* vol. 69, *(13),* pp. 5601-5609, 2009. Available: https://pubmed.ncbi.nlm.nih.gov/19549916 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2853876/. DOI: 10.1158/0008-5472.CAN-08-3860.

[107] K. Al-Nedawi *et al*, "Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells," *Nat. Cell Biol.,* vol. 10, *(5),* pp. 619-624, 2008. Available: https://pubmed.ncbi.nlm.nih.gov/18425114. DOI: 10.1038/ncb1725.

[108] G. Taraboletti *et al*, "Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells," *The American Journal of Pathology,* vol. 160, *(2),* pp. 673-680, 2002. Available: https://pubmed.ncbi.nlm.nih.gov/11839588 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1850663/. DOI: 10.1016/S0002-9440(10)64887-0.

[109] S. S. Sidhu *et al*, "The microvesicle as a vehicle for EMMPRIN in tumor–stromal interactions," *Oncogene,* vol. 23, *(4),* pp. 956-963, 2004.

[110] J. Wolfers *et al*, "Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming," *Nat. Med.,* vol. 7, *(3),* pp. 297-303, 2001. Available: https://pubmed.ncbi.nlm.nih.gov/11231627. DOI: 10.1038/85438.

[111] W. Chen *et al*, "Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells," *Eur. J. Immunol.,* vol. 36, *(6),* pp. 1598-1607, 2006. Available: https://pubmed.ncbi.nlm.nih.gov/16708399. DOI: 10.1002/eji.200535501.

[112] A. Bobrie *et al*, "Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression," *Cancer Res.,* vol. 72, *(19),* pp. 4920-4930, 2012. Available: https://pubmed.ncbi.nlm.nih.gov/22865453. DOI: 10.1158/0008-5472.CAN-12-0925.

[113] X. Xiang *et al*, "TLR2-mediated expansion of MDSCs is dependent on the source of tumor exosomes," *The American Journal of Pathology,* vol. 177, *(4),* pp. 1606-1610, 2010. Available: https://pubmed.ncbi.nlm.nih.gov/20802178 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2947257/. DOI: 10.2353/ajpath.2010.100245.

[114] C. Liu *et al*, "Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function," *Journal of Immunology (Baltimore, Md.: 1950),* vol. 176, *(3),* pp. 1375-1385, 2006. Available: https://pubmed.ncbi.nlm.nih.gov/16424164. DOI: 10.4049/jimmunol.176.3.1375.

[115] A. J. Abusamra *et al*, "Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis," *Blood Cells Mol. Dis.,* vol. 35, *(2),* pp. 169-173, 2005. Available: https://pubmed.ncbi.nlm.nih.gov/16081306. DOI: 10.1016/j.bcmd.2005.07.001.

[116] C. Grange *et al*, "Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche," *Cancer Res.,* vol. 71, *(15),* pp. 5346-5356, 2011. Available: https://pubmed.ncbi.nlm.nih.gov/21670082. DOI: 10.1158/0008-5472.CAN-11-0241.

[117] A. Hoshino *et al*, "Tumour exosome integrins determine organotropic metastasis," *Nature,* vol. 527, *(7578),* pp. 329-335, 2015. Available: https://pubmed.ncbi.nlm.nih.gov/26524530 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4788391/. DOI: 10.1038/nature15756.

[118] B. Costa-Silva *et al*, "Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver," *Nat. Cell Biol.,* vol. 17, *(6),* pp. 816-826, 2015. Available: https://pubmed.ncbi.nlm.nih.gov/25985394 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5769922/. DOI: 10.1038/ncb3169.

[119] H. Peinado *et al*, "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET," *Nat. Med.,* vol. 18, *(6),* pp. 883-891, 2012. Available: https://pubmed.ncbi.nlm.nih.gov/22635005 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3645291/. DOI: 10.1038/nm.2753.

[120] C. L. A. Yeung *et al*, "Exosomal transfer of stroma-derived miR21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1," *Nature Communications,* vol. 7, pp. 11150, 2016.

[121] Y. Xu *et al*, "Macrophages transfer antigens to dendritic cells by releasing exosomes containing dead-cell-associated antigens partially through a ceramide-dependent pathway to enhance CD4(+) T-cell responses," *Immunology,* vol. 149, *(2),* pp. 157-171, 2016. Available: https://pubmed.ncbi.nlm.nih.gov/27278624 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5011679/. DOI: 10.1111/imm.12630.

[122] L. Zitvogel *et al*, "Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes," *Nat. Med.,* vol. 4, *(5),* pp. 594-600, 1998. Available: https://doi.org/10.1038/nm0598-594. DOI: 10.1038/nm0598-594.

[123] L. A. Smyth *et al*, "CD73 expression on extracellular vesicles derived from CD4 CD25 Foxp3 T cells contributes to their regulatory function," *Eur. J. Immunol.,* vol. 43, *(9),* pp. 2430-2440, 2013.

[124] Z. Deng *et al*, "Exosomes miR-126a released from MDSC induced by DOX treatment promotes lung metastasis," *Oncogene,* vol. 36, *(5),* pp. 639-651, 2017. Available: https://doi.org/10.1038/onc.2016.229. DOI: 10.1038/onc.2016.229.

[125] M. Roh-Johnson *et al*, "Macrophage-Dependent Cytoplasmic Transfer during Melanoma Invasion In Vivo," *Developmental Cell,* vol. 43, *(5),* pp. 549-562.e6, 2017. Available: http://www.sciencedirect.com/science/article/pii/S1534580717309073. DOI: https://doi.org/10.1016/j.devcel.2017.11.003.

[126] E. Segura, S. Amigorena and C. Théry, "Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses," *Blood Cells Mol. Dis.,* vol. 35, *(2),* pp. 89-93, 2005. Available: https://pubmed.ncbi.nlm.nih.gov/15990342. DOI: 10.1016/j.bcmd.2005.05.003.

[127] M. F. S. Lindenbergh and W. Stoorvogel, "Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells," *Annu. Rev. Immunol.,* vol. 36, *(1),* pp. 435-459, 2018. Available: https://doi.org/10.1146/annurev-immunol-041015-055700. DOI: 10.1146/annurev-immunol-041015-055700.

[128] C. Pizzirani *et al*, "Stimulation of P2 receptors causes release of IL-1beta-loaded microvesicles from human dendritic cells," *Blood,* vol. 109, *(9),* pp. 3856-3864, 2007. Available: https://pubmed.ncbi.nlm.nih.gov/17192399. DOI: 10.1182/blood-2005-06-031377.

[129] Z. Cai *et al*, "Immunosuppressive exosomes from TGF-β1 gene-modified dendritic cells attenuate Th17-mediated inflammatory autoimmune disease by inducing regulatory T cells," *Cell Res.,* vol. 22, *(3),* pp. 607-610, 2012.

[130] S. Kim *et al*, "Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis," *The Journal of Immunology,* vol. 174, *(10),* pp. 6440-6448, 2005.

[131] V. R. Simhadri *et al*, "Dendritic cells release HLA-B-associated transcript-3 positive exosomes to regulate natural killer function," *PloS One,* vol. 3, *(10),* pp. e3377, 2008. Available: https://pubmed.ncbi.nlm.nih.gov/18852879 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2566590/. DOI: 10.1371/journal.pone.0003377.

[132] S. Viaud *et al*, "Dendritic cell-derived exosomes promote natural killer cell activation and proliferation: a role for NKG2D ligands and IL-15Ralpha," *PloS One,* vol. 4, *(3),* pp. e4942, 2009. Available: https://pubmed.ncbi.nlm.nih.gov/19319200 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2657211/. DOI: 10.1371/journal.pone.0004942.

[133] S. Munich *et al*, "Dendritic cell exosomes directly kill tumor cells and activate natural killer cells via TNF superfamily ligands," *Oncoimmunology,* vol. 1, *(7),* pp. 1074-1083, 2012. Available: https://pubmed.ncbi.nlm.nih.gov/23170255 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3494621/. DOI: 10.4161/onci.20897.

[134] C. T. Guy, R. D. Cardiff and W. J. Muller, "Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease." *Mol. Cell. Biol.,* vol. 12, *(3),* pp. 954-961, 1992.

[135] M. C. Jamur and C. Oliver, "Permeabilization of cell membranes," in *Immunocytochemical Methods and Protocols*Anonymous 2010.

[136] K. Hua and R. J. Ferland, "Fixation methods can differentially affect ciliary protein immunolabeling," *Cilia,* vol. 6, *(1),* pp. 5, 2017. Available: https://doi.org/10.1186/s13630-017-0045-9. DOI: 10.1186/s13630-017-0045-9.

[137] A. Vernay and P. Cosson, "Immunofluorescence labeling of cell surface antigens in Dictyostelium," *BMC Research Notes,* vol. 6, pp. 317, 2013. Available: https://pubmed.ncbi.nlm.nih.gov/23937900 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3751103/. DOI: 10.1186/1756-0500-6-317.

[138] M. Surman *et al*, "Deciphering the role of ectosomes in cancer development and progression: focus on the proteome," *Clin. Exp. Metastasis,* vol. 34, *(3-4),* pp. 273-289, 2017. Available: https://pubmed.ncbi.nlm.nih.gov/28317069 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5442264/. DOI: 10.1007/s10585-017-9844-z.

[139] S. Wan *et al*, "CD8α+ CD11c+ Extracellular Vesicles in the Lungs Control Immune Homeostasis of the Respiratory Tract via TGF-β1 and IL-10," *The Journal of Immunology,* vol. 200, *(5),* pp. 1651-1660, 2018.

[140] N. Ismail *et al*, "Macrophage microvesicles induce macrophage differentiation and miR-223 transfer," *Blood,* vol. 121, *(6),* pp. 984-995, 2013. Available: https://doi.org/10.1182/blood-2011-08-374793. DOI: 10.1182/blood-2011-08-374793.

[141] M. Yang *et al*, "Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells," *Molecular Cancer,* vol. 10, *(1),* pp. 117, 2011. Available: https://doi.org/10.1186/1476-4598-10-117. DOI: 10.1186/1476-4598-10-117.

[142] D. C. Ayre *et al*, "CD24 induces changes to the surface receptors of B cell microvesicles with variable effects on their RNA and protein cargo," *Scientific Reports,* vol. 7, *(1),* pp. 8642, 2017. Available: https://doi.org/10.1038/s41598-017-08094-8. DOI: 10.1038/s41598-017-08094-8.

[143] S. Runz *et al*, "Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM," *Gynecol. Oncol.,* vol. 107, *(3),* pp. 563-571, 200 . DOI: S0090-8258(07)00647-6 [pii].

[144] F. Menay *et al*, "Exosomes Isolated from Ascites of T-Cell Lymphoma-Bearing Mice Expressing Surface CD24 and HSP-90 Induce a Tumor-Specific Immune Response," *Frontiers in Immunology,* vol. 8, pp. 286, 2017. Available: https://www.frontiersin.org/article/10.3389/fimmu.2017.00286. DOI: 10.3389/fimmu.2017.00286.

[145] S. Keller *et al*, "CD24 is a marker of exosomes secreted into urine and amniotic fluid," *Kidney Int.,* vol. 72, *(9),* pp. 1095-1102, 2007. DOI: S0085-2538(15)52799-3 [pii].

[146] R. Li *et al*, "Roles of Arf6 in cancer cell invasion, metastasis and proliferation," *Life Sci.,* vol. 182, pp. 80-84, 2017. DOI: S0024-3205(17)30294-1 [pii].

[147] T. Tsuruyama *et al*, "Histology of intestinal allografts: lymphocyte apoptosis and phagocytosis of lymphocytic apoptotic bodies are diagnostic findings of acute rejection in addition to crypt apoptosis," *Am. J. Surg. Pathol.,* 2012.

[148] G. Kristiansen *et al*, "CD24 expression is a new prognostic marker in breast cancer," *Clinical Cancer Research,* vol. 9, *(13),* pp. 4906-4913, 2003.

[149] H. Singh-Jasuja *et al*, "The mouse dendritic cell marker CD11c is down-regulated upon cell activation through Toll-like receptor triggering," *Immunobiology,* vol. 218, *(1),* pp. 28-39, 2013. DOI: S0171-2985(12)00036-8 [pii].

[150] C. H. GeurtsvanKessel and B. N. Lambrecht, "Division of labor between dendritic cell subsets of the lung," *Mucosal Immunology,* vol. 1, *(6),* pp. 442-450, 2008. Available: https://doi.org/10.1038/mi.2008.39. DOI: 10.1038/mi.2008.39.

[151] T. C. Badea, Y. Wang and J. Nathans, "A noninvasive genetic/pharmacologic strategy for visualizing cell morphology and clonal relationships in the mouse," *Journal of Neuroscience,* vol. 23, *(6),* pp. 2314-2322, 2003.

[152] M. D. Muzumdar *et al*, "A global double-fluorescent Cre reporter mouse," *Genesis,* vol. 45, *(9),* pp. 593-605, 2007.

[153] A. Vaezi *et al*, "Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium," *Developmental Cell,* vol. 3, *(3),* pp. 367-381, 2002.

[154] K. Nguyen-Ngoc *et al*, "3D culture assays of murine mammary branching morphogenesis and epithelial invasion," in *Tissue Morphogenesis* 2015.

Curriculum Vitae

Luda Lin

jimlin1668478052@hotmail.com| llin46@jhu.edu | +1 (215) 452-1005

Education

Johns Hopkins University Aug 2018–May 2020

MSE in Biomedical Engineering | 2-year Thesis-based | Regenerative and Immune Engineering

The Hong Kong Polytechnic University Sept 2014–Aug 2018

BSc in Biomedical Engineering with 1st honours | Medical Instrument and Sensors

University of Pennsylvania Sept–Dec 2017

Exchange in Bioengineering for one semester

Research Experience

Interaction Between Natural Killer Cells and Breast Cancer Cells During Tumor Invasion

Johns Hopkins University | PI: Dr. Andrew Ewald Jan 2019– May 2020

I have been participating in the project of researching on how natural killer cells (NK cells), an important member of the innate immune system, control breast cancer metastasis, led by Dr. Isaac Chan in Ewald Lab.

My project is 1) to research on detailed mechanisms of NK-tumor cell interaction via ligand-receptor binding and secretion of soluble factors in the mouse model, 2) to establish human NK-tumor coculture models of invasion and growth, with the breast tumor cell line of MCF-7 and MDA-MB-231 and the NK cell line of NK-92 by adapting original mouse coculture models and 3) to investigate interaction between metastases and other immune cell types such as dendritic cells.

I learned how to dissect a mouse and to acquire organoids from breast tumors collected from the mouse. I learned the tumor organoid invasion assay in collagen gels, tumor colony formation assay in Matrigel, and NK-tumor coculture assay with both gels. I developed those assays with human breast cancer cell lines with the drop hanging method I learned in my previous research. I learned to use both DIC and confocal microscopes, and their time-lapse recording functions. I learned immunocytochemistry, immunohistochemistry and flow cytometry. I learned to use a cryostat. I learned to use a high content imager and a slice scanner. I learned to use both CellEvent Caspase-3/7 reporter and TUNEL assay to observe apoptosis. I also did ELISA assay and plenty of Western blot. I also learned to do ELISA. I also learned to do tail vein injection.

Observation of Invasion Modes of Liver Cancer Jan–Jul 2017

The Hong Kong Polytechnic University | PI: Dr. Youhua Tan

This was my capstone project in the final year of the undergraduate university. The aim of the project was originally to research on the roles of cancer stem cells (CSCs) and non-cancer stem cells during tumor invasion. The hepatocellular carcinoma cell line Huh-7 were used as the model.

I developed the 3D collagen gel tumor invasion model independently in the lab according to instructions of certain papers only. I learned to form spheroids from cell suspension via drop-hanging, which were transferred to collagen gel and were observed daily for at most 7 days. Modes of morphological changes happened to those spheroids, including invasion modes, were collected. Finally, I learned to transfect cancer cells with Lipo3000, and trying to tell whether CSCs or non-CSCs take the lead during tumor invasion by observing chimera spheroids formed with labeled non-CSCs and unlabeled CSCs through a fluorescence microscope.

Physical Properties of Cancer Cells on Substrate with Different Viscoelasticity

University of Pennsylvania | PI: Dr. Paul Janmey Oct–Dec 2017

I worked to help a master student in research on physical properties of cancer cells on substrate with different viscoelasticity. I learned to make polyacrylamide gels with modulated viscoelasticity. My main work was to do cell tracing with ImageJ with cancer cells cultured on substrate with different viscoelasticity sent by the master student.

Ultrasound Stimulation on MSCs for Recovery from Bone Fracture Jan–July 2016

The Hong Kong Polytechnic University | Supervisor: Dr. Chunyi Wen

I worked to help a master student under Dr. Wen in a project about ultrasound stimulation on MSCs for recovery from bone fracture. I learned to passage mesenchymal stem cells (MSCs) and to transfect quantom dots. We established an experiment system with a water tank and an ultrasound generator. The aim of the research was to see whether quantom dots would be transferred to nuclei after stimulation by ultrasound.

Work Experience

Reagent Research and Design Intern June– Aug 2017

the Biochemical Reagent R&D Department, Mindray Medical Co., Shenzhen, China

- Worked as an online maintenance intern in China's largest medical equipment manufacturer;
- Utilized experiment methods and root cause analysis to solve complaints about biochemical reagents and autoanalyzers from clients;
- Gathered and analyzed data from imported database from complainers in Microsoft Excel to find out causes of complaints;
- Detected limits of blank and limits of detection of several reagents in certain types of autoanalyzers;

Market Analyst Intern Sept– Nov 2016

TF Security, Shenzhen, China

- analyzed the market of medical devices in China;
- Finished a market analysis report on the orthopedic market in China;

Skills

Software: R language, MATLAB, Mathematica, ImageJ, GraphPad Prism, Chem3D, Microsoft Office (Word and Excel)

Awards

- Dean's Honours List of Faculty of Engineering for 3 consecutive years in the Hong Kong Polytechnic University (2014/2015, 2015/2016, 2016/2017)
- Outstanding Student Award of the Department of Biomedical Engineering 2018 - The Hong Kong Polytechnic University

Academic Interests

I am interested in how heterogeneous components of a tumor, including different subpopulations of cancer cells and non-cancer cells and extracellular matrices, interact with and influence each other, during tumor development, leading to invasion and metastasis. Dr. Kenneth Pienta describes cancer as a social dysfunction within a cell community, which is a description that I could not agree more.

Then, I would like to participate in development of cellular immunotherapy against cancer, which is to use knowledge of cell-cell interaction as mentioned above to medicine. I am interested in development of proteins with domains of expected functions and gene modification via CRISPR-Cas9.

Thirdly, I would like to do research on stem cell lineage determination via mechanical cues. In this field, I am most interested in the finding that mechanical cues only can lead to reprogramming of differentiated cells into stem cells.